TESIS DOCTORAL

STUDY AND CHARACTERIZATION OF THE *Ph1* LOCUS AS A TOOL TO PROMOTE INTERSPECIFIC CHROMOSOME ASSOCIATIONS BETWEEN WHEAT AND BARLEY SPECIES

Autora:

Dña. María Dolores Rey Santomé Licenciada en Biología- Consejo Superior de Investigaciones Científicas- Universidad de Córdoba

Directora:

Dr. Dña. Pilar Prieto Aranda Dra. en Biología- Científica titular- Consejo Superior de Investigaciones Científicas

Presentada para la obtención del Título de

DOCTORA CON MENCIÓN INTERNACIONAL POR LA UNIVERSIDAD DE CÓRDOBA

Departamento de Mejora Genética

MARZO 2015

TITULO: *Study and characterization of the Ph1 locus as a tool to promote interspecific chromosome associations between wheat and barley species*

AUTOR: *Maria Dolores Rey Santomé*

© Edita: Servicio de Publicaciones de la Universidad de Córdoba. 2015
Campus de Rabanales
Ctra. Nacional IV, Km. 396 A
14071 Córdoba

www.uco.es/publicaciones
publicaciones@uco.es

TÍTULO DE LA TESIS

STUDY AND CHARACTERIZATION OF THE *Ph1* LOCUS AS A TOOL TO PROMOTE INTERSPECIFIC CHROMOSOME ASSOCIATIONS BETWEEN WHEAT AND BARLEY SPECIES

DOCTORANDA: Dña. María Dolores Rey Santomé

INFORME RAZONADO DE LA DIRECTORA DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como trabajos y publicaciones derivados de la misma)

Dra. Dña. PILAR PRIETO ARANDA, Científica titular del Consejo Superior de Investigaciones Científicas informa que,

El presente trabajo se ha realizado dentro del Programa de Doctorado "Biociencias y Ciencias Agroalimentarias" de la Universidad de Córdoba. Previamente, la doctoranda realizó el máster "Producción, Protección y Mejora Vegetal" en el 2011.

La tesis se ha llevado a cabo dentro de un proyecto europeo del *European Research Council* (ERC-Starting Grants ref. 243118) del que soy investigadora principal. Este trabajo supone un avance en el conocimiento del locus *Ph1* durante la meiosis en trigo. El locus *Ph1* se ha usado como

herramienta genética para promover asociaciones interespecíficas entre cromosomas homeólogos de trigo harinero y cebada silvestre o cultivada.

Esta tesis doctoral ha dado lugar a las siguientes publicaciones científicas:

Artículos en revistas SCI

- Rey MD, Prieto P (2014) Dynamics of DNA replication during premeiosis and early meiosis in wheat. PLoS ONE 9, (10) e107714 DOI: 10.1371/journal.pone.0107714. Publicado en Plos one. *Plos one* pertenece al primer cuartil en el área de las ciencias multidisciplinares (IF 3.534 y posición 8 / 55)

- Rey MD, Calderón MC, Prieto P. The use of the *ph1b* mutant to induce recombination between the chromosomes of wheat and barley. En revisión en Frontiers in Plant Science. *Frontiers in Plant Science* pertenece al primer cuartil en el área de ciencias vegetales (IF 3.638 y posición 23/199)

- Rey MD, Calderón MC, Rodrigo MJ, Zacarías L, Alós E, Prieto P. Use of wheat *ph1b* mutant to introgress *Hordeum chilense* chromosome fragments to enhance carotenoid content in bread wheat. En revisión en Journal of Experimental Botany. *Journal of Experimental Botany* pertenece al primer cuartil en el área de ciencias vegetales (IF 3.638 y posición 11/199)

- Rey MD, Prieto P. A rapid assay to detect alien genetic introgressions in bread wheat by dot-blot hybridization. En proceso.

Artículos en revistas SCI (ajenas a la tesis doctoral)

La doctoranda ha colaborado en otros trabajos realizados en mi laboratorio, y como resultado, ha participado como coautora en un artículo científico:

- Calderón MC, Rey MD, Cabrera A, Prieto P (2014) Subtelomeric regions play a key role in chromosome recognition and pairing during meiosis in wheat. Publicado en Scientific reports 4: 6488 DOI: 10.1038/srep06488. *Scientific reports* pertenece al primer cuartil en el área de las ciencias multidisciplinares (IF 5.078 y posición 5 / 55).

Además durante su estancia predoctoral se incorporó activamente al grupo del Prof. Moore en el John Innes Center, Norwich Research Park, donde ha trabajado con marcadores moleculares Kaspar para mapear una región de *Aegilops sharonesis* responsable de los genes gametocidas que provocan roturas cromosómicas en el fondo genético del trigo harinero. El trabajo ha dado lugar a un artículo científico en el que la doctoranda participa como coautora:

- Knight E, Binnie A, Draeger T, Moscou M, Rey MD, Sucher J, Mehra S, King I, Moore G. Mapping the "breaker" element of the gametocidal locus proximal to a block of sub-telomeric heterochromatin on the long arm of chromosome 4**S^{sh}** of *Aegilops sharonensis.* Aceptado en Theoretical and Applied Genetics. *Theoretical and Applied Genetics* pertenece al primer cuartil en el área de ciencias vegetales (IF 3.506 y Posición 24/ 199).

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

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 (Japan), del 08 al 14 de Septiembre de 2013. Comunicación: 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), del 09 al 13 de Junio de 2013. Comunicación: Oral
- Rey MD, Calderón MC, Prieto P. Chromosome pairing between wheat and *Hordeum chilense* chromosomes is promoted in the background of *ph1* mutants. Plant and Animal Genome XXI. San Diego (California), del 12 al 16 de Enero del 2013. Comunicación: Póster

Congresos nacionales

- Rey MD, Calderón MC, Prieto P. Obtención de líneas de interés entre *Hordeum chilense* y trigo harinero en el fondo genético del mutante *ph.* VIII Seminario de Citogenética. Alcalá de Henares, Madrid (España), del 24 al 26 de Junio de 2014. Comunicación: Oral
- Rey MD, Prieto P. Estudio del apareamiento cromosómico entre especies del género *Hordeum* y trigo harinero en el fondo genético de mutantes *ph1*. VI Congreso de Mejora Genética de Plantas. Gijón (España), del 11 al 13 de Septiembre de 2012. Comunicación: Oral

Rey MD, Prieto P. Desarrollo de líneas de sustitución y de adición de *Hordeum chilense* y *Hordeum vulgare* en el fondo genético de mutantes de apareamiento de trigo harinero. I Congreso Científico de Investigadores en Formación en Agroalimentación / II Congreso

Científico de Investigadores en Formación (Nacional), del 08 al 09 de Mayo del 2012. Comunicación: Oral. En este congreso la doctoranda recibió un premio a la mejor comunicación en la rama Agroalimentaria.

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

Córdoba, 06 de Febrero de 2015

Firma de la directora:

Fdo.: PILAR PRIETO

TÍTULO DE LA TESIS

STUDY AND CHARACTERIZATION OF THE *Ph1* LOCUS AS A TOOL TO PROMOTE INTERSPECIFIC CHROMOSOME ASSOCIATIONS BETWEEN WHEAT AND BARLEY SPECIES

DOCTORANDA

Dña. Mª Dolores Rey Santomé

ESCRITO RAZONADO DEL RESPONSABLE DE LA LÍNEA DE INVESTIGACIÓN **(Ratificando el informe favorable del director. Sólo cuando el director no pertenezca a la Universidad de Córdoba).**

La doctoranda ha desarrollado un trabajo de mejora genética de trigo utilizando los mutantes de apareamiento ph1b para promover asociaciones cromosómicas entre las cebadas silvestre y cultivada y el trigo harinero. Además, ha profundizado en el conocimiento del papel del locus Ph1 en la meiosis de trigo, concretamente cómo afecta a la replicación durante los estadios iniciales de la meiosis. Sus trabajos de investigación han dado lugar a publicaciones en revistar internacionales (una publicada, otra aceptada relacionada con su estancia en el John Innes Centre de Norwich, otra enviada y varias en preparación), además de comunicaciones orales en congresos internacionales, presentadas por la propia doctoranda.

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

Córdoba, 04 de Febrero de 2015

Firma del responsable de línea de investigación

Fdo. Antonio Martín Muñoz

GENERAL INDEX

Note: This current PhD Thesis is written in a bilingual format as a requirement for the International Mention

AGRADECIMIENTOS/ ACKNOWLEDGEMENTS

Quisiera dedicar unas palabras a todas aquellas personas que de una manera u otra me han acompañado y facilitado su consejo y ánimo a lo largo de este proceso, sin las cuales no hubiera sido posible lograr este objetivo.

En primer lugar agradecer a las entidades que han financiado esta tesis doctoral ya que sin ellas esta tesis no habría sido posible. Al proyecto europeo ERC-Starting Grant-243118, al ministerio de Economía y Competitividad por la concesión del proyecto AGL2012-33264 y al Fondo Europeo de Desarrollo Regional (FEDER). Al Instituto de Agricultura Sostenible, y en especial al departamento de Mejora Genética Vegetal.

De igual manera mi más sincero agradecimiento a mi Directora, la Dr. Pilar Prieto, a quien debo el realizar el doctorado. Gracias por ofrecerme sus valiosos conocimientos y experiencia profesional y por toda la paciencia tenida en mí.

En especial a mis compañeras de laboratorio a las cuales de alguna manera u otra les tengo que agradecer todo su apoyo, ánimo y colaboración en todo momento. A Melania y a Enri por sus conocimientos y gran apoyo. A María José por hacerme ver siempre el lado positivo de las cosas. A Carmen por sus locuras y apoyo en todo momento. Gracias por haberme sacado una sonrisa tanto en los buenos como en los malos momentos.

A Graham por darme la oportunidad de realizar una estancia en su laboratorio y a todas las personas del John Innes Centre, quienes hicieron

mi estancia mucho más amena. Gracias por todos los conocimientos adquiridos en esos tres meses.

Agradecer, también, a los revisores de mi tesis doctoral, Ken-Ichi Nonomura y Paul Hoskisson, por su rapidez y por sus interesantes comentarios.

A todos mis amigos/as de Cádiz, de Córdoba y de la escuela oficial de idiomas que me han ayudado a desconectar y disfrutar de todos los ratos pasados juntos.

Agradecer hoy y siempre a mi familia porque a pesar de no estar presentes físicamente, siempre han estado detrás de cualquier llamada telefónica para pedirles consejos. A mis padres por su confianza, por apoyarme en todas las decisiones que he tomado en mi vida y especialmente por enseñarme a luchar por todo aquello que quiero. A mi *sister* quien siempre me apoyó y animó a continuar cuando parecía que me iba a rendir.

A Antonio, por ser la persona que ha compartido el mayor tiempo a mi lado, porque en su compañía las cosas malas se convierten en buenas. Le doy las gracias por haberme hecho creer cada día que podía hacerlo, por toda su ayuda y, principalmente, por hacerme feliz.

A todos, ¡muchas gracias!

Mª Dolores Rey Santomé

SUMMARY

Interspecific hybridization is used to introduce desirable characters from related species into allopolyploids such as wheat. The development of substitution and addition lines of related species in bread wheat can be useful as a genetic tool to transfer agronomic traits in the background of bread wheat. However, there is a low level of pairing and recombination between wheat chromosomes and those from the relative species. Different approaches have been carried out to promote pairing and recombination between related chromosomes such as the *ph1b* mutant, ionizing radiations and gametocidal genes. However, the most important discovery has been the *Ph1* locus which regulates pairing and recombination between homologous chromosomes during meiosis in wheat. Thus, in its absence, related chromosomes could associate and recombine.

Chapter 2 of this work examines the dynamic of DNA replication during premeiosis and early meiosis in wheat using flow cytometry, which has allowed the quantification of the amount of DNA in wheat anther in each stage meiotic stage. Chromosome replication was detected in wheat during premeiosis and early meiosis until the stage of pachytene, when chromosomes are associated in pairs to further recombine and correctly segregate in the gametes. Also, an important role of the *Ph1* locus on the length of meiotic DNA replication in wheat was shown using flow cytometry.

Chapter 3 deals with chromosome manipulation to induce meiotic recombination between barley and wheat in the absence of the *Ph1* locus. Genetic crosses between the *ph1b* mutant and both wild and cultivated barley substitution and addition lines in wheat were carried out. More than

800 plants were generated and screened for the barley introgressions using molecular markers and *in situ* hybridization. The use of the *ph1b* mutant facilitated chromosome pairing and interspecific recombination between (wild and cultivated) barley and wheat to develop genetic introgressions from those species into wheat. A detailed description of the wheat breeding program was described.

Chapter 4 explains the development and characterization of 7$\mathbf{H}^{ch}$-bread wheat translocation lines enriched in carotenoids using the wheat *ph1b* mutant. Both 7$\mathbf{H}^{ch}$α·7**A**L and 7**A**S·7$\mathbf{H}^{ch}$β translocation lines were characterized by *in situ* hybridization and using molecular markers. In addition, a HPLC analysis revealed that both disomic translocation lines presented double carotenoid content than wheat. The *Psy1* gene, which is involved in carotenoid synthesis, was also cytogenetically mapped on the 7$\mathbf{H}^{ch}$α chromosome arm and a proteomic analysis confirmed that the presence of chromosome 7$\mathbf{H}^{ch}$ introgressions in wheat did not alter the proteomic profile of the wheat flour.

Chapter 5 describes a rapid assay to detect small random alien genetic introgressions from relative species in the wheat background. The robustness of this technique was confirmed by *in situ* hybridization.

In summary, this work sheds some light on the role of the *Ph1* locus during meiosis in wheat. The *Ph1* affects replication during early meiosis. In addition, the absence of the *Ph1* did induce a low but significative level of chromosome pairing and recombination between *Hordeum* sp. species and wheat chromosomes. All the plant material generated in this thesis will serve as potential donor material for wheat breeding.

RESUMEN

La hibridación interespecífica se ha utilizado para introducir caracteres de interés desde especies relacionadas al trigo harinero. El desarrollo de líneas de adición y de sustitución de especies relacionadas en trigo harinero es una herramienta clave para introducir genes de interés en el fondo genético del trigo harinero. Como especies relacionadas se utilizaron *Hordeum chilense* y *Hordeum vulgare*, que presentan genes de interés agronómico para la mejora genética del trigo. Sin embargo, el nivel de apareamiento y recombinación entre el trigo y especies relacionadas es muy bajo. Existen diferentes métodos para promover eventos de recombinación entre trigo y especies relacionadas, tales como la radiación iónica, los genes gametocidas y el locus *Ph1*. Sin embargo, el locus *Ph1* es el descubrimiento más importante ya que regula el apareamiento y la recombinación entre cromosomas homólogos en meiosis en trigo. En su ausencia, cromosomas relacionados pueden asociarse en pares y recombinar.

El capítulo 2 de esta tesis doctoral estudia la dinámica de la replicación del ADN durante la premeiosis y los estadios tempranos de la meiosis en trigo usando citometría de flujo. Además, se estudió el efecto del locus *Ph1* mostrando un papel importante en la duración de la replicación meiótica del ADN en el trigo harinero.

El capítulo 3 trata de la manipulación cromosómica para inducir recombinación entre las cebadas silvestre y cultivada y el trigo harinero en ausencia del locus *Ph1*. El uso de un mutante *ph1b* facilitó el apareamiento cromosómico y la recombinación interespecífica entre ambas cebadas y el trigo. Se obtuvieron más de 800 plantas a partir de los cruzamientos genéticos entre el mutante *ph1b* y líneas de adición y de

sustitución de cebada silvestre y cebada cultivada. Todas estas plantas se analizaron mediante marcadores moleculares e hibridación *in situ*.

El capítulo 4 se centra en el desarrollo y caracterización de líneas de translocación del cromosoma 7$\mathbf{H^{ch}}$ en trigo harinero enriquecidas en carotenoides. Los cruzamientos genéticos entre las líneas de sustitución de cromosoma 7 de *H. chilense* en trigo y el mutante *ph1b* permitieron el desarrollo de líneas de traslocación para ambos brazos del cromosoma 7$\mathbf{H^{ch}}$. Estas translocaciones se caracterizaron mediante hibridación *in situ* y marcadores moleculares. Ambas líneas presentaron doble cantidad de carotenoides que el trigo harinero. Además, se localizó citogenéticamente el gen *Psy1* en el brazo corto del cromosoma 7$\mathbf{H^{ch}}$, y se confirmó que la presencia de las introgresiones del cromosoma 7$\mathbf{H^{ch}}$ no alteraba el perfil proteómico de la harina de trigo.

Finalmente, en el quinto capítulo se optimizó un método rápido y económico para detectar introgresiones de especies relacionadas en trigo harinero. Esta técnica permite realizar un rápido y fiable escrutinio para detectar pequeños fragmentos de otras especies en trigo harinero introducidas durante un programa de mejora genética.

En resumen, este trabajo aporta conocimiento al papel que desempeña el locus *Ph1* durante la meiosis en trigo. El locus *Ph1* afecta la replicación durante los estadios iniciales de la meiosis y en su ausencia se ha inducido un bajo pero significativo nivel de apareamiento y recombinación entre los cromosomas de *Hordeum* y del trigo harinero. Todo el material vegetal generado en esta tesis podría utilizarse como donador genético en programas de mejora genética del trigo harinero.

Chapter I

Introduction and objectives

Wheat production

More than one billion people have insufficient food to sustain life and food supply might not be enough to meet this expected human demand by 2050. Genetics in agriculture is a tool that can be used as part of the solution to this challenge. Wheat, maize and rice are the three major crops; all together cover 40% of the 1.4 billion ha global crop land (FAO-STAT, 2009). The world production of wheat reached 690 million tons in 2012−2013, being the European Union (20% of the total) the largest producer followed by China (18%) and India (14%) (FAO-STAT 2013; http://faostat.org). With a predicted world population of 9 billion in 2050, the demand for wheat is expected to increase by 60%. To meet this demand, annual wheat yield increases must rise from the current level of below 1% to at least 1.6%. All countries share the need to improve wheat yield, tolerance to abiotic stresses, pathogens and pests, as well as to improve input use efficiency for a more sustainable wheat production. Better agronomic practices and development of innovative cropping systems are also a priority.

Origin, evolution and classification of wheat

Wheat has been part of the economic and cultural development of the human race. It is considered a basic food for human consumption, although much is used for animal feeding. Since the beginning of agriculture, approximately 10 000 years ago, cereals have provided the main source of calories for people. Wheat and barley, two members of the *Triticeae* tribe have been particularly important because they served as the principal grain stock that enabled the founding of agriculture in the Middle East and led to its successful spread around the word (Zohary and Hopf, 2000). The *Triticeae* tribe is an unusual group of plants which includes diploids, such as barley and rye, tetraploids such as durum wheat, hexaploids such as bread wheat, spelt and triticale, which can

also be found at the octoploid genetic level. Probably, einkorn wheat (*Triticum monococcum*) was the first wheat specie to be widely cultivated 10 000 years ago in south eastern Turkey (Feuillet *et al.*, 2008). All species share the same basic set of seven chromosomes, mostly in diploid form. The first evolutionary event was hybridization of diploid wheat closely related to *Triticum urartu* whose genomic constitution was **AA** with a yet unknown species from the Sitopsis section that provided the **B** genome and was closely related to *Aegilops speltoides* (**SS**). This fertile tetraploid (**AABB**) was domesticated and became known as emmer wheat or *T. turgidum* (Luo *et al.*, 2007). This new cereal is more vigorous, has a higher yield and more broadly adapted to different environmental conditions that its progenitors. A second evolutionary event that led to the bread wheat lineage occurred when durum wheat was crossed with *A. tauschii* whose genomic constitution was **DD**, wild diploid specie. A double of the chromosomes in gametes gave hexaploid species with an **AABBDD** genome, known as *T. aestivum* or bread wheat. Bread wheat is included in the Poaceae family, commonly known as grasses, in which some of the most cultivated varieties are *T. durum* and *T. compactum*. However, *T. aestivum* is the bread grain most important in the world.

One key factor in the success of wheat as a global food crop is its adaptability to a wide range of climatic condition due to, in part, its allohexaploid genome. With 21 pairs of chromosomes, bread wheat is structurally an allopolyploid with three homoeologous (related) sets of seven pairs of chromosomes in each of the **A**, **B** and **D** genomes (Figure 1). Homoeologous chromosomes have a similar linear sequence of genes but a different repetitive content, while homologues (identical chromosomes) have the same linear sequence of genes and repetitive content. Genetically, wheat behaves as a diploid during meiosis because homoeologous pairing is prevented through the action of *Ph* (*Pairing homologous*) genes (Martinez-Pérez *et al.*, 2001). Each genome is about 5.5 Gb

in size and carries, in addition to related sets of genes, a high proportion (>80%) of highly repetitive transposable elements (TEs) (Eilam *et al.*, 2007; Wicker *et al.*, 2011). To date, relatively little is known about the position and distribution of genes on each of the bread wheat chromosomes and their evolution during the polyploidization events that resulted in the emergence of the hexaploid species.

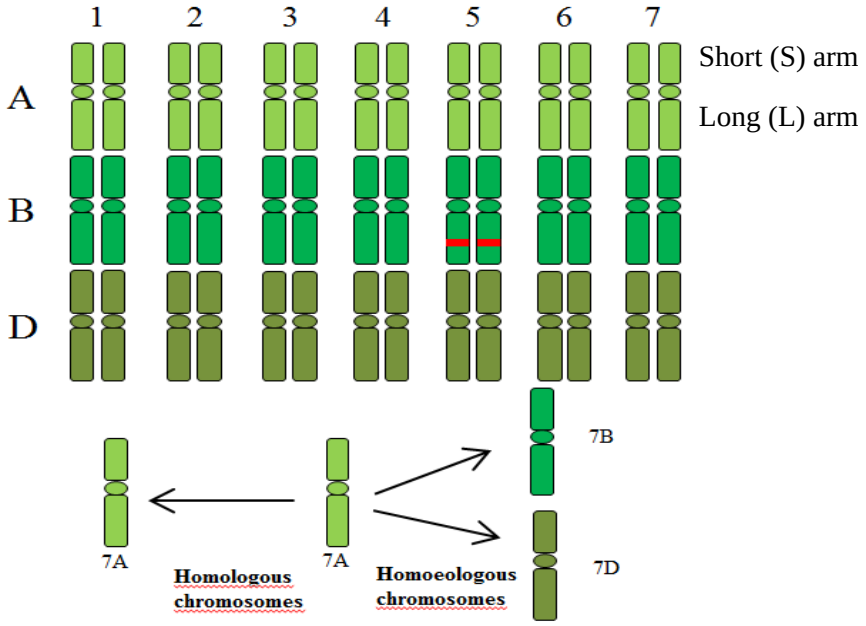

Figure 1. Bread wheat genome (2n=2x=42) composed of three related genomes (**A**, **B** and **D**) and showing the *Ph1* locus (see section 3) on chromosome 5**B**L.

Meiosis in bread wheat

Meiosis is a highly conserved process in eukaryotes and occupies a central role in the life cycle. Meiosis differs from mitosis in that a single round of DNA replication precedes two sequential cell divisions, so that an initially diploid cell generates four haploid cells. Before meiosis begins, each chromosome is replicated. Initiation of meiosis can be cytologically recognized at the leptotene

stage, chromosome condensation begins and the installation of axial elements along the chromosomes is completed (Zickler and Kleckner, 1999). Homologous chromosomes previously distributed throughout the nucleus (Bass *et al.*, 2002; Maestra *et al.*, 2002) must approach and recognize each other to enter into intimate contact and form bivalents. In many organisms, a cluster of telomeres occurs, telomeres attach to the nuclear envelop and congregate to form a cluster, the meiotic bouquet (Bass *et al.*, 2000; Niwa *et al.*, 2000; Trelles-Sticken *et al.*, 2000, Cowan *et al.*, 2001; Scherthan, 2001; Harper *et al.*, 2004). This structure facilitates in some way chromosome sorting, homologous recognition and the association of homologous chromosomes into pairs. Simultaneously, centromeres associated at the opposite nuclear pole in an extension of the interphase Rabl configuration (Anamthawat-Jonsson and Heslop-Harrison, 1990; Marshall *et al.* 1996; Abranches *et al.* 1998). Rabl configuration is maintained until the completion of homologue association at the end of zygotene. At the onset of meiosis, chromosomes undergo conformational changes (Dawe *et al.*, 1994; Colas *et al*., 2008). Homologous chromosomes can only pair when they are in the same conformational state and the signal to initiate the process occurs at the same time in the two homologues (Prieto *et al.*, 2004). A fluorescence *in situ* hybridization analysis of chromosome arrangement in hexaploid wheat showed that centromeres associate prior to meiosis, usually in pairs (Aragón-Alcaide *et al.*, 1997a; Martinez-Pérez *et al.*, 1999, 2003). At the beginning of the zygotene stage, telomeres aggregate and chromosome pairing and synaptonemal complex (SC) formation is initiated distally (Holm, 1977; Prieto *et al*. 2004a). From zygotene until pachytene homologous chromosomes pair and synapse. In diplotene the homologues are held together as bivalents through the regions where crossovers and recombination occurred (Petronczki *et al.*, 2003; Page and Hawley, 2004). If a crossover occurs, the recombination event matures into a chiasma, which is visible at diplotene. There is almost always one crossover per chromosome arm

and per meiosis to guarantee proper alignment of bivalents on the equatorial plate of the first meiotic division and subsequent proper disjunction of homologous chromosomes. The chromosomes condense further and detach from the nuclear envelope during diakinesis. Finally, homologous chromosomes separate at anaphase I. The segregation of homologous pairs of chromosomes at the first division is therefore dependent on their prior pairing, synapsis, and recombination at earlier stages. The second meiotic division is equational and separates sister chromatids of each chromosome to give rise to the haploid gametes.

It is estimated that approximately 70% of plants are polyploids (Masterson, 1994). The process of homologue recognition and pairing in meiosis is more complicated in polyploids due to the greater number of related chromosomes. All allopolyploids must behave as diploids during meiosis to produce viable and fertile gametes. Three or more chromosome sets, either from a species (autopolyploid) or from related diploid species that sexually hybridized (allopolyploid), coexist in polyploids plants. At meiosis, more than two homologous or genetically related (homoeologous) chromosomes can compete for synapsis and recombination. Pairing of homoeologous chromosomes would result in multivalent associations and improper segregation of chromosomes at anaphase I to produce unbalanced and unviable gametes. To avoid this problem, many allopolyploids show a diploid-like meiotic behaviour with strict homologous pairing (Sears, 1976). Little is known about the mechanism that allows the discrimination between homologous and homoeologous pairing. In bread wheat it was proposed that the association of homologous chromosomes before meiosis prevents multivalent association and allows a true diploid-type homologous pairing (Aragón-Alcaide *et al.*, 1997; Martinez-Pérez *et al*., 2003). The *Ph1* locus is the most studied mechanism to avoid pairing between homoeologous chromosomes in bread wheat. This locus is located on the long

arm of chromosome 5**B** in wheat (Okamoto, 1957; Riley and Chapman, 1958; Sears and Okamoto, 1958; Sears, 1977). The *Ph1* locus has been recently describe as a 2.5 Mb region containing a segment of subtelomeric heterochromatin inserted into a cluster of cdk-2 related genes, which share some similarities to mammalian Cdk2 (Griffiths *et al.*, 2006; Al-kaff *et al.*, 2008; Yousafzai *et al.*, 2010). Cdks are a group of Ser/Thr protein kinases that control the progression through the various phases of the cell cycle and subsequent cell divisions in eukaryotic cells. Cdks has been shown to participate in the premeiotic replication, chromatin condensation, transcription of the earliest meiotic gene (*Asy1*), homologue pairing/synapsis, resolution of incorrect pairing at pachytene and recombination. Precisely, the *Ph1* 5**B** locus contains seven kinase-like genes and its homoeologous loci on chromosomes 5**A** and 5**D** contain five and two kinase-like genes, respectively. When the chromosome 5**B** is absent, the other homoeologous chromosomes 5**A** and 5**D** seem to compensate its loss and increment the content of Cdk causing chromosomal imbalance leading to recognition of homoeologous chromosomes. However, the mode of action of the *Ph1* locus remains still unknown. In addition, the absence of the *Ph1* locus cannot prevent homoeologous chromosomes from associating via their centromeres when homologous are absent in the hexaploid wheat-rye hybrid (Martinez-Pérez *et al.*, 2001). This suggests the discrimination between homologous and homoeologous chromosomes does not occur initially but is performed after chromosomes alignment (Calderón *et al*., 2014).

Interspecific hybrids between related species and bread wheat

There are several related species from *Hordeum*, *Secale*, and *Aegilops* that can be used to introduce desirable agronomic genes into bread wheat. However, this PhD thesis is only focused in the *Hordeum* genus. *Hordeum chilense* is an extremely polymorphic diploid wild barley from South of America. It has a high crossability with other members of the *Triticeae* tribe and presents several

agronomical characteristics which could be transferred into wheat, such as high carotenoid content on chromosome 7**H**^{ch} (Alvarez *et al.*, 1998), genes of resistance to *Septoria tritici* on chromosome 4**H**^{ch} and in a minor extend by chromosome 5**H**^{ch} (Rubiales *et al.*, 1992, 2000), genes controlling tolerance to salt (NaCl) on chromosome 5**H**^{ch} (Forster *et al.*, 1990), resistance genes to powdery mildew (*Erysiphe graminis* f. sp. *tritici*) on chromosomes 1**H**^{ch} and 7**H**^{ch} (Rubiales *et al.*, 1993, 2001) and resistance genes to common bunt (*Tilletia caries*) on chromosome 7**H**^{ch} and to a minor extent by chromosome 6**H**^{ch} (Rubiales and Martín, 1999).

On the other hand, *H. vulgare* (cultivated barley) has great genetic diversity for tolerating many biotic and abiotic stresses which can be used for wheat breeding. Several resistance genes have been characterized in *H. vulgare* (Qi *et al.*, 1996), for example, some powdery mildew (caused by *Blumeria graminis* f. sp. *hordei*) resistance genes are located on chromosome 1**H**, five genes (*Mlra*, *Mla*, *Mlk* and *Mlnn* genes) on 1**H**S, which also carries leaf rust (caused by *Puccinia hordei)* resistance gene called *Rph4*, and one gene (*MlLa* gene) on chromosome 1**H**L. *Rps4* gene located on chromosome 1**H** conferring resistance to stripe rust (caused by *P. striiformis*). There are two leaf rust resistance genes located on chromosome 2**H**, *Rph1* and *Rph16*. Finally, two genes conferring resistance to scald (caused by *Rhynchosporium secalis*) are located on chromosome 3**H**. Related to abiotic stresses, there are important genes as *Alp* responsible for aluminum tolerance (Tang *et al.*, 2000; Wang *et al.*, 2007) and *int-c* responsible for lateral spikelet fertility (Komatsuda and Mano, 2002) located on barley chromosome 4. Chromosome 1**H** is of special interest because it carries several interesting genes for wheat breeders, but unfortunately, at present, barley substitution or addition lines for chromosome 1**H** in wheat are not available to be used in a wheat breeding program. These lines have not been produced yet due to several genes present on the long arm of this chromosome

which cause extreme cytological abnormalities at meiosis resulting in sterility (Islam *et al.*, 1981; Islam and Shepherd, 1990). However an addition line for this chromosome carrying a pair of heteromorphic 1**H** chromosomes with a pair of 6**H** chromosomes were produced (Islam and Shepherd, 2000).

Sexual hybridization between an allopolyploid and a wild relative generally produces an interspecific hybrid containing a haploid set of the allopolyploid and wild relative chromosomes. In some cases, there is a low level of pairing and recombination between wheat and wild relative chromosomes. Diploid, tetraploid and hexaploid wheats have been crossed with species of the genus *Aegilops*, *Agropyron*, *Secale*, *Haynaldia*, *Hordeum* and *Elymus*, and several intergeneric hybrids have been produced. The first hybrid, Triticale, was generated in Scotland in 1876 from wheat and rye, although the first fertile hybrid was obtained in 1938 (Oettler, 2005). The close relationship between *Hordeum* and wheat chromosomes enables the generation of fertile amphiploid hybrids between the cultivated species of this tribe and also between the cultivated species and their wild relatives. Several hybrids and amphiploids between *Hordeum* and wheat have been developed. The first amphiploid between *H. chilense* and *T. aestivum* (cv. Chinese Spring (CS) was obtained by Martín and Chapman in 1977, but fertility of this octoploid tritordeum (2n = 8x = 56, $\mathbf{H^{ch}H^{ch}AABBDD}$) was extremely low.

Wheat-alien hybridization makes possible to transfer agronomically useful genes from one species into another. Several alien genes transfer have been reported from *Secale cereale* L. into wheat, but very few studies have dealt with the development of wheat/barley translocation lines. The effect of the *ph1b* mutant was used to promote recombination from the chromosomes 4**H** and 5**H** wheat-barley addition lines produced by Islam *et al.*, 1981 (Sherman *et al.*, 2001), translocation and recombinant lines which confer resistance to stem rust

(Liu *et al.*, 2011) and 4**V**S chromosome recombinants to map wheat yellow mosaic virus resistance gene from *Haynaldia villosa* (Zhao *et al.*, 2013).

Others different methods have been used to transfer genetic material from wild relatives into bread wheat, such as ionizing radiations or gametocidal genes. The ionizing radiation treatment, frequently used to produce hybrids that carry a small portion of the alien genome as individual chromosomes or translocations, was first used by Sears, 1956 to induce chromosome breaks and transfer genes from *Aegilops umbellulata* to wheat. Related to the gametocidal (Gc) system, the presence of Gc genes from *Aegilops* causes chromosome breakage in bread wheat gametes in monosomic condition (Endo, 1988, 1990, 2007). This system has been used to produce barley dissection lines of common wheat carrying rearranged barley chromosomes from wheat-barley addition lines (Shi and Endo, 1997, 1999, 2000). Both methods cause random chromosomes breaks in all chromosomes. Thus, since the discovery of the *Ph1* locus, it has been used widely and in wheat to induce homoeologous recombination (Sears, 1977). The development of introgressions is carried out by sexual hybridization to bring the wild or alien genome into the wheat cultivated background and homologues or homoeologous recombination to eliminate the deleterious alleles or genes, a phenomenon known as "linkage drag". In fact, this phenomenon has limited the efficient exploitation of wild relatives in breeding programs because the process to eliminate the negative alleles is tedious and time consuming. Reducing the size of an introgressed chromosomal segment relies on recombination; however, recombination is not distributed homogeneously along the chromosomes of wheat and barley and depends strongly on the level of sequence similarity. Comparative sequencing between homoeologous sequences in wheat and between cultivars in barley (Scherrer *et al.*, 2005) has shown that sequence similarity is restricted mainly to the genic area and that the intergenic regions

are highly divergent (Wicker *et al.*, 2003; Gu *et al.*, 2004; Chantret *et al.*, 2005; Gu *et al.*, 2006).

Molecular markers and cytogenetic techniques, such as fluorescence *in situ* hybridization (FISH) and genomic *in situ* hybridization (GISH), facilitate the process of analyzing alien introgressed segments (Calderón *et al.*, 2012; Zhao *et al.*, 2013). Perhaps the best example of introgression of chromatin from a relative into wheat is the 1**B**L/1**R**S chromosomal translocation (Zeller, 1973; Zeller and Hsam, 1984). The 1**R**S chromosome from rye carries several genes whose protein products increase grain yield providing race-specific disease resistance to major rust diseases (*Lr29*/*Yr26* leaf and yellow rust resistance genes), improved adaptation and stress tolerance and higher kernel weight (Zarco-Hernández *et al.*, 2005). Other example includes the introgression of functional alleles of the *Pin* genes, whose protein products are responsible for endosperm texture in wheat from the diploid wheat *T. monococcum* ($\mathbf{A^mA^m}$) (Giroux and Morris, 1998).

Objectives

General objective

The aim of this work is to go deeper into the knowledge of the effect of the *Ph1* locus during early meiosis in wheat, and the use of the *ph1b* mutant to promote interspecific chromosome associations between bread wheat chromosomes and those from relative species such as *Hordeum chilense* and *Hordeum vulgare.*

Specific objectives

1. **Determine the effect of the *Ph1* locus in meiotic replication in wheat** (*Chapter II*). Anthers of bread wheat in the presence and in the absence of the *Ph1* locus will be used to study DNA replication during premeiosis and early meiosis by flow cytometric analysis and *in situ* hybridization.

2. **Development of *H. chilense* and *H. vulgare* addition and substitution lines in wheat in the absence of the *Ph1* locus to promote chromosome pairing between wheat and both *H. chilense* and *H. vulgare* chromosomes in the background of the *ph1* mutant** (*Chapter III*). *Hordeum chilense* substitution and *H. chilense* and *H. vulgare* addition lines in bread wheat will be used as parental lines in genetic crosses with the wheat line deficient for the *Ph1* locus. Bread wheat *ph1b* mutants will be checked for the *ph1b* deletion using the ABC_{920} SCAR marker and the presence of a *Hordeum* chromosome will be analyzed using microsatellite and EST markers. In addition, *in situ* hybridization will be used to identify *H. chilense* and *H. vulgare* introgression and translocation lines in the background of *ph1b* mutant.

3. **Development and characterization of *H. chilense* chromosome $7H^{ch}$ translocation lines in bread wheat for carotenoid enrichment** (*Chapter IV*). Wheat-*Hordeum chilense* robertsonian translocation lines ($7\mathbf{H}^{ch}\alpha{\cdot}7\mathbf{A}$L and $7\mathbf{A}$S$\cdot7\mathbf{H}^{ch}\beta$) will be obtained using the *ph1b* mutant in the wheat background and will be characterized using microsatellite markers and *in situ* hybridization. The carotenoid content of these translocations lines will be evaluated and the *Psy1* gene, which is involved in of the carotenoid biosynthetic pathway, will be cytogenetically mapped on chromosome $\mathbf{7H}^{ch}$**.**

4. **Development of a rapid and user-friendly assay to detect small alien genetic introgressions in bread wheat** (*Chapter VI*). A dot blot assay will be set up to rapid and reliable screen small random alien genomic introgressions introduced during a wide breeding program in the background of bread wheat.

Chapter II

Dynamics of DNA replication during premeiosis and early meiosis in wheat

Published in:

Rey MD, Prieto P. Dynamics of DNA replication during premeiosis and early meiosis in wheat. PLoS One (2014) 9: e107714

Abstract

Meiosis is a specialised cell division that involves chromosome replication, two rounds of chromosome segregation and results in the formation of the gametes. Meiotic DNA replication generally precedes chromosome pairing, recombination and synapsis in sexually developing eukaryotes. In this work, replication has been studied during premeiosis and early meiosis in wheat using flow cytometry, which has allowed the quantification of the amount of DNA in wheat anther in each phase of the cell cycle during premeiosis and each stage of early meiosis. Flow cytometry has been revealed as a suitable and user-friendly tool to detect and quantify DNA replication during early meiosis in wheat. Chromosome replication was detected in wheat during premeiosis and early meiosis until the stage of pachytene, when chromosomes are associated in pairs to further recombine and correctly segregate in the gametes. In addition, the effect of the *Ph1* locus, which controls chromosome pairing and affects replication in wheat, was also studied by flow cytometry. Here we showed that the *Ph1* locus plays an important role on the length of meiotic DNA replication in wheat, particularly affecting the rate of replication during early meiosis in wheat.

Keywords: flow cytometry, wheat, *Ph1* locus, DNA replication, chromosome pairing, meiosis, cell cycle

Introduction

Meiosis is a specialized type of cell division common to sexually developing eukaryotes that generates four haploid gametes from a single diploid cell. The evolutionary trends of cell cycle including DNA replication, growth control and cell division are mechanistically well conserved among eukaryotes (Stillman, 1996; Gutierrez, 1998; Huntley and Murray, 1999; Mironov *et al.*, 1999; Sherr *et al.*, 1999). During the cell cycle, proliferating cells pass through four stages: G1, the cell growths and the nucleus has a 2C DNA content (where C is the DNA content of a haploid genome with chromosome number n); S, DNA replicates (2C → 4C); G2, a second growth period during which the nucleus retains a 4C content until the last phase; and M, mitosis or meiosis in somatic or germinal cells, respectively, when genetic material is divided into two daughter nuclei (4C → 2C). During meiosis a second division occurs and four haploid cells (gametes) are finally obtained from one initial diploid cell.

Duplication of the genome during S phase of the cell cycle is a highly organized process, usually followed in germinal cells by chromosome pairing of homologous (identical) chromosomes, recombination and synapsis (Forsburg, 2002). Pre-meiotic DNA replication has been shown to be similar to pre-mitotic S phase in many aspects (Collins and Newlon, 1994; Simchen, 1974; Gallert and Sipiczki, 1991) although several important features distinguish meiotic from mitotic replication, including the trigger that initiates the process (Forsburg and Hodson, 2000). In addition, pre-meiotic S phase is on average 2-3 times longer than pre-mitotic S-phase in all organisms studied (Bennet and Smith, 1972; Holm, 1977; Cha *et al.*, 2000), probably because necessary interactions between homologues for their successful recombination and segregation are initiated during pre-meiotic S phase (Forsburg, 2002; Smith, *et al.* 2001; Jorgan, 2006). Additional periods of DNA synthesis have also been reported during early meiosis in leptotene, zygotene and pachytene (Wimber

and Prensky, 1963; Lima-De-Faria *et al.*, 1966; Mukherjee and Cohen, 1968). In fact, detection of replication during early meiosis was essential for understanding the mechanism of crossing-over during recombination (Hotta and Stern, 1961; Lima-de-Faria *et al.*, 1968).

Pre-meiotic replication has been found to be connected to later events occurring in meiosis such as recombination and reductional chromosome segregation (Watanabe *et al.*, 2001; Strich, 2004). Moreover, replication has also been shown to be closely connected temporally to chromosome condensation at the onset of meiosis (Drouin *et al.*, 1991). Most of the studies about pre-meiotic replication have been conducted in yeast (Zegerman and Diffley, 2007) and little is known about meiotic replication in plants. Replication has been recently studied during early meiosis in wheat-rye hybrids in the presence and in the absence of the *Ph1* locus (Greer *et al.*, 2012). Wheat (*Triticum aestivum* L.) is a staple food for most of the world population, and understanding its genetics and genome organization is of great value for genetics and plant breeders. The *Ph1* locus controls homologous chromosome pairing in wheat (Okamoto, 1957; Riley and Chapman, 1958; Sears and Okamoto, 1958; Sears, 1977), and has been defined to a cluster of kinase-like genes containing a segment of heterochromatin (Griffiths *et al.*, 2006; Al-kaff *et al.*, 2008). Cyclin dependent kinases (CDKs) play an important role in the cell cycle regulation and transcription control (Harper and Adams, 2001). The *Ph1*-like gene in wheat shares some homology to Cdk2 in mammals, which regulates the progression of replication through controlling chromatin decondensation during S phase (Alexandrow and Hamlin, 2005). In wheat, the *Ph1*-like gene regulates premeiotic replication, chromatin condensation, transcription of the earliest meiotic gene (*Asy1*), homologue pairing/synapsis, resolution of incorrect pairing at pachytene and recombination (Yousafzai *et al.*, 2010). Recent studies have described that the *Ph1* locus may affect replication through either an increment

in the activation of origins and hence the rate of replication of the dispersed chromatin or, a delay in the initiation of heterochromatin replication in the absence of the *Ph1* locus (Greer *et al.*, 2012).

Flow cytometry has become a useful method for studying the characteristics of eukaryotic cells, with applications in crop and horticultural science (Leus *et al.*, 2009). Although flow cytometry has been crucial for chromosome sorting, allowing sequencing in species with large genomes such as wheat (Vrána *et al.*, 2000), other popular flow cytometric applications are the measurement of cellular DNA content for studies of ploidy, mostly in plants, and the identification of the cell distribution during the cell cycle (Bino *et al.*, 1993; Dolezel and Bartos, 2005; Brito *et al.*, 2010). In fact, cell cycle-phase distribution of the DNA synthesis activity can be effectively determined by flow cytometry after isolation of nuclei. The four distinct phases (G1-, S-, G2- and M) can be recognised in a proliferating cell population by flow cytometry, although G2- and M-phase, which both have an identical DNA content (4C), cannot be discriminated based only on their differences in DNA content. Therefore cytogenetic approaches are required to determine whether chromosomes have entered meiosis by visualizing chromosome condensation and pairing.

In this chapter we aimed to further our knowledge of pre-meiotic and meiotic replication in wheat, focusing in the early meiosis stages using flow cytometry. To achieve this, we established a quick and user-friendly flow cytometry-based method to investigate replication during meiosis in wheat through the quantification of the amount of DNA in each meiotic stage. Flow cytometry has been revealed as a rapid and robust method to quantify the amount of DNA during the five sub-stages (leptotene, zygotene, pachytene, diplotene and diakinesis) of early meiosis (prophase I) in wheat, and allowed a correlation

between the amount of DNA and the level of replication at each stage during early meiosis in bread wheat. In addition, the effect of the *Ph1* locus on the timing and on the rate of replication during early meiosis in wheat is also discussed.

Materials and Methods

Plant material

Seeds of bread wheat (*Triticum aestivum* L., 2n = 2x = 42) cv. Chinese Spring in the presence and in the absence of the *Ph1* locus were kindly provided by Dr. Steve Reader from The John Innes Centre (JIC, Norwich, U.K.). DNA from wheat lines either in the presence or in the absence of the *Ph1* locus were extracted from young frozen leaf tissue using the CTAB method (Murray and Thompson, 1980) with some modifications (Hernández *et al.*, 2001). CS and CS *ph1b* mutants were checked for the *ph1b* deletion using the ABC_{920} SCAR marker as described previously (Wang *et al.*, 2002).

Seeds were germinated in the dark at 25ºC on moistened filter paper in petri dishes for 2 days and then transferred into pots and grown in the greenhouse at 26ºC during the day and 22ºC at night with a photoperiod of long days (16 h of daylight).

Preparation of samples for flow cytometry and *in situ* hybridization

Spikes were collected from plants entering meiosis, and fixed in 100% ethanol: acetic acid (3:1, v/v) for at least one week. Florets from fixed wheat spikes were checked under a phase-contrast microscope (PrimoStar light microscope; Carl Zeiss, Göttingen, Germany) for correct assignment of the meiotic stage. Each floret has three synchronous anthers, thus one anther per floret was squashed in 45% acetic acid in water and assigned to each meiotic stage by observation under a PrimoStar light microscope (Carl Zeiss, Göttingen, Germany). The two remaining anthers were fixed in 100% ethanol: acetic acid 3:1 (v/v) and used for

flow cytometric analysis and *in situ* hybridization. Young leaves from both wheat lines were used as somatic control in flow cytometry experiments.

In situ hybridization

Fixed anthers were squashed in 45% acetic acid in water for *in situ* hybridization. The telomeric sequence was amplified by PCR using the (5'-TTTAGGG-3') and (5'- CCCTAAA-3') primers in the absence of template DNA (Cox *et al.*, 1993) and a cereal centromeric sequence (CCS1) was amplified using the conditions described by (Aragón-Alcaide *et al.*, 1996). The *in situ* hybridization protocol was performed according to (Prieto *et al.*, 2004). Digoxigenin-labelled centromeres and biotin-labelled telomeres were detected with antidigoxigenin-FITC (Roche Applied Science, Indianapolis, IN, USA) and streptavidin- Cy3 conjugates (Sigma, St. Louis, MO, USA), respectively. Chromosomes were counterstained with DAPI (4', 6-diamidino-2-phenylindole) and mounted in Vectashield. Hybridization signals were visualized using a Nikon eclipse 80i epifluorescence microscope. Images were captured with a Nikon CCD camera using the Nikon 3.0 software (Nikon Instruments Europe BV, Amstelveen, The Netherlands) and processed with Photoshop 4.0 software (Adobe Systems Inc., San Jose, California, USA).

Flow cytometric analysis

Wheat anthers at each meiotic stage were ground with a pestle in 400 µl nuclear extraction buffer of the Partec CyStain® UV Precise T kit (PARTEC GmbH, Münster, Germany) for 3 min at room temperature following the instructions of the supplier. The suspension was filtered through a 30 µm nylon mesh filter to discard cell debris. Finally, each sample was stained with 4', 6-diamidino-2-phenylindole (DAPI) for 1 min to measure the amount of nuclear DNA using a CyFlow® Ploidy Analyser (PARTEC GmbH, Münster, Germany) equipped with an UV LED, located at the University of Cordoba (Dpt. of Genetics). Three

independent experiments, consisting of the measurement of the DNA content of samples in leptotene, zygotene, pachytene, diplotene, diakinesis and metaphase I either in the presence or in the absence of the *Ph1* locus, were carried out on different days. In addition, 3 replicate measurements of each sample were taken for each biological replicate. At least 5000 nuclei were counted in each sample either in the presence or in the absence of the *Ph1* locus and the coefficient of variation (CV) for each sample was always under 8.0%. The histograms were analyzed using the Cylchred Software from Cardiff University developed by Terry Hoy, which is a cell cycle analysis software based on previously developed algorithms (Ormerod *et al.*, 1987), and allows removing the cell debris marker from the histograms.

Statistical analyses

Statistical analyses were performed using STATISTIX 9.0 software (Analytical Software, Tallahassee, FL, USA). The analysis of variance (ANOVA) was based on randomized blocks. Means were separated using the Least Significant Difference (LSD) test with a probability level of 0.05.

Results

Identification, isolation and flow cytometric analysis of meiocytes during premeiosis and early meiosis in wheat

To study the progression of replication during early meiosis in wheat, one anther per floret was carefully checked to determine the meiotic stage using a light microscopy. Since all the anthers in the same flower are synchronized, the two remaining anthers were stored in 100% ethanol: acetic acid (3:1, v/v) at 4ºC. The identification, selection and isolation of anthers was carried out until a total of 150 anthers were accumulated in each meiosis stage of prophase I (leptotene, zygotene, pachytene, diplotene and diakinesis) and metaphase I, either in the presence or in the absence of the *Ph1* locus. Each sample was then

separated in three aliquots of 50 anthers each with the aim of having three independent replicates of each stage of meiosis, either in the presence or in the absence of the *Ph1* locus for three independent experiments. In addition, three different flow cytometric measurements were taken from each sample in each experiment to account for equipment deviations.

Flow cytometric determination of the nuclear DNA content in a wheat anther sample, either in the presence or in the absence of the *Ph1* locus, was distributed in a histogram with two peaks corresponding to G0/G1 phases (un-replicated cells; 2C DNA content) and G2/M phases (replicated cells; 4C DNA content), respectively (Figures 1 and 2). As expected, most of the cells in each meiosis stage were identified in the 2C peak (G0/G1) for all the samples analysed (Figures 1 and 2). The small peak (4C) corresponded to those cells that had already finished replication and cells going under active replication were detected between the 2C and 4C peaks (Figures 1 and 2).

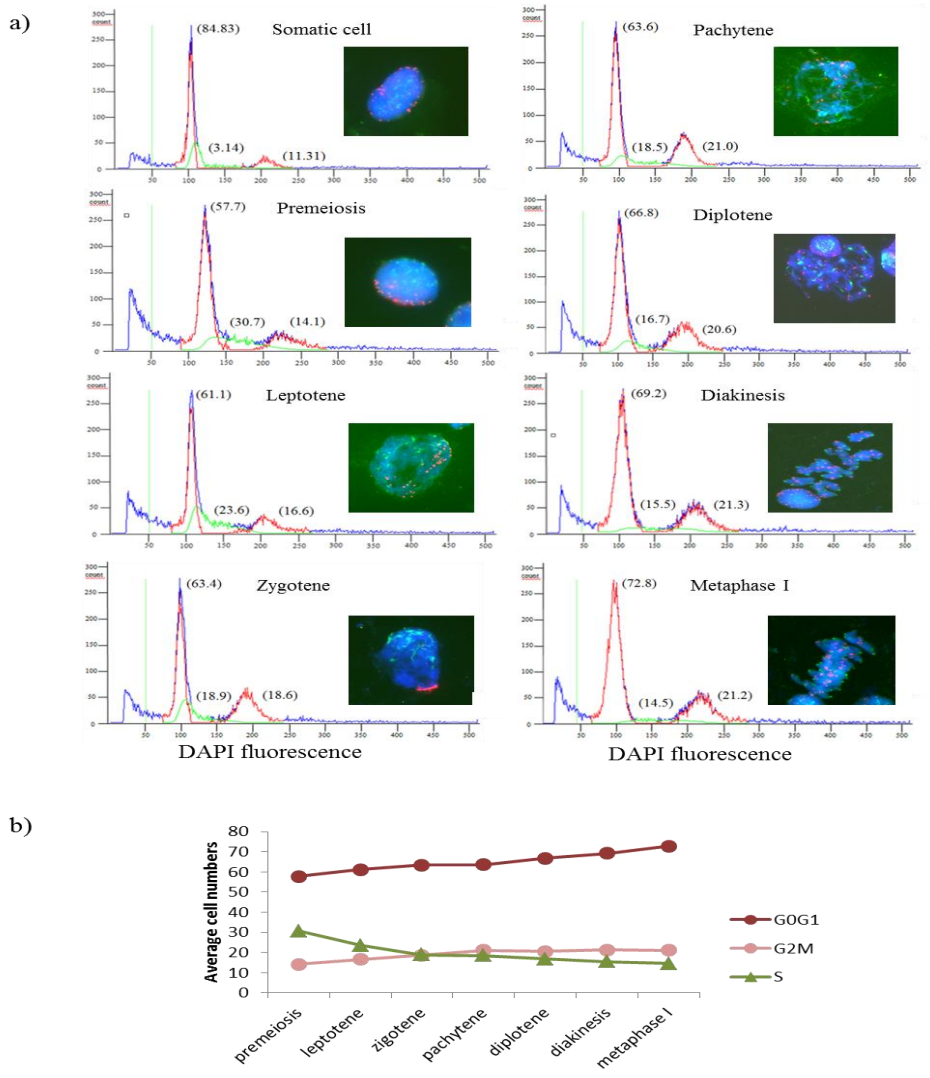

Figure 1. Flow cytometric analysis of DNA replication in wheat anthers during early meiosis in the presence of the *Ph1* locus. A. Flow cytometric histograms of the nuclear DNA content of isolated anther nuclei in each stage of meiosis, which was determined by the number and organization of centromeres (green) and telomeres (red) using fluorescence *in situ* hybridization. The percentage of cells in G0/G1, S and G2/M are in brackets. G0/G1 and G2/M phases are shown in red, S phase is shown in green and total histogram (measured as total nuclei) is shown in blue. B. Progression of the percentage of cells in G0/G1, S and G2/M phases during early meiosis.

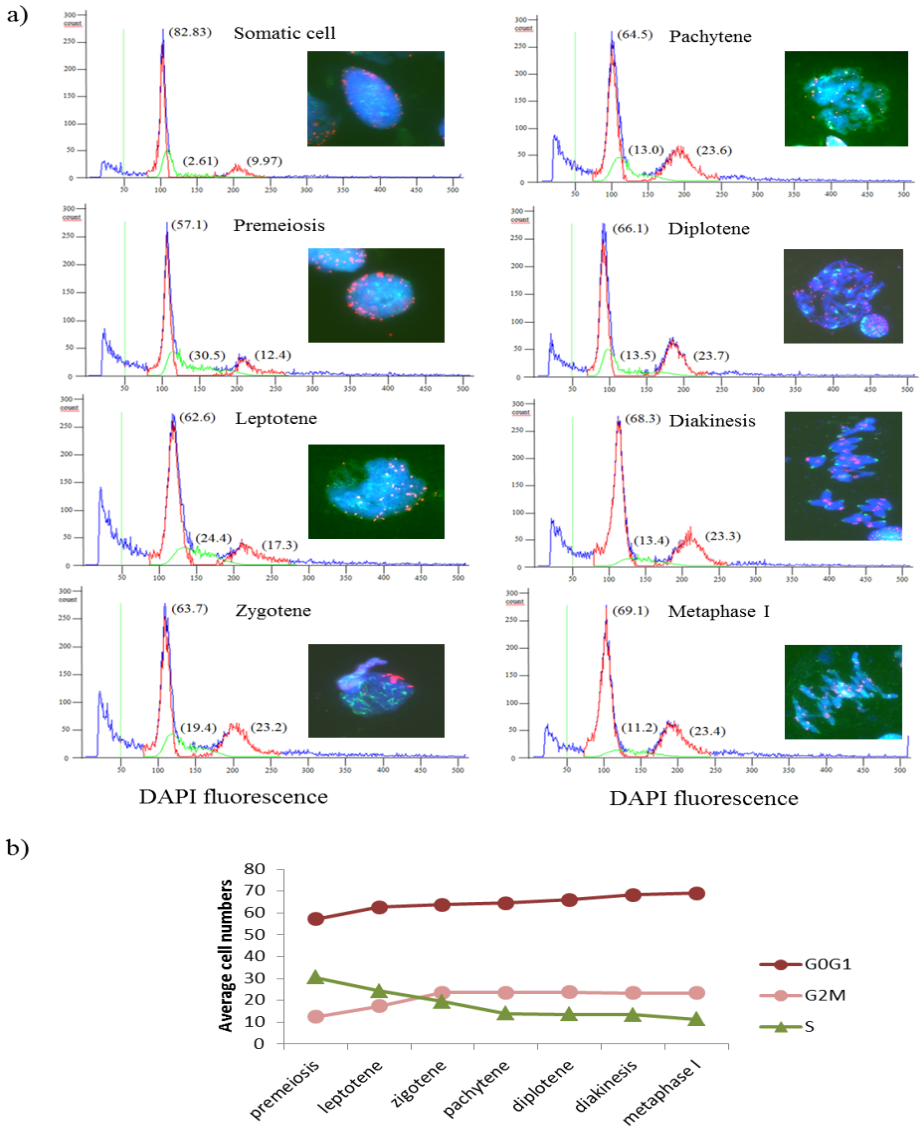

Figure 2. Flow cytometric analysis of DNA replication in wheat anthers during early meiosis in the absence of the *Ph1* locus. A. Flow cytometric histograms of the nuclear DNA content of isolated anther nuclei in each stage of meiosis, which was determined by the number and organization of centromeres (green) and telomeres (red) using fluorescence *in situ* hybridization. The percentage of cells in G0/G1, S and G2/M are in brackets. G0/G1 and G2/M phases are shown in red, S phase is shown in green and total histogram (measured as total nuclei) is shown

in blue. B. Progression of the percentage of cells in G0/G1, S and G2/M phases during early meiosis.

The amount of DNA during the cell cycle in somatic tissues was always measured by flow cytometry at the beginning and at the end of each experiment for both wheat lines (*Ph1*+ and *Ph1*-), to have a basal reference of unreplicated cells, replicated cells and somatic replication of each wheat line and, in addition, to monitor instrument or staining variations (Figures 1 and 2). As expected, most of the cells were in G0/G1 stage and no significant differences were found in wheat somatic tissue in the presence and in the absence of the *Ph1* locus in any case (Table 1). The replication value obtained for the somatic tissue (3.1 ± 0.2 and 2.6 ± 0.6 in *Ph1*+ and *Ph1*- wheat lines, respectively) was always at least five times lower than the minimum replication value obtained in early meiosis (15.5 ± 1.2 and 13.5 ± 0.1 in diakinesis in *Ph1*+ and *Ph1*- wheat lines, respectively) (Tables 2 and 3).

	G0/G1 phase		**S phase**		**G2/M phase**	
	Mean ± SE	CV	Mean ± SE	CV	Mean ± SE	CV
Ph1+	84.8 ± 4.1a	4.8	3.1 ± 0.2a	3.5	11.3 ± 0.4a	3.8
Ph1-	82.8 ± 3.1a	3.7	2.6 ± 0.6a	3.9	10.0 ± 0.6a	5.8

Table 1. Flow cytometric determination of the percentage distributions of wheat somatic nuclei in each phase of the cell cycle in the presence and in the absence of the *Ph1* locus (*Ph1*+ and *Ph1*-, respectively). G0/G1 and G2/M values correspond to un-replicated and post-replicated cells, respectively. S phase values correspond to cells under active replication. Values are given as a mean of 9 measures, standard error of the mean (SE) and coefficient of variation (CV). The same letter indicates that there is no difference among treatments (*Ph1*+ and *Ph1*-) within the same cell cycle phase (G0/G1, G2/M and S) in somatic cells at $P<0.05$.

	G0/G1 phase		S phase		G2/M phase	
	Mean ± SE	CV	Mean ± SE	CV	Mean ± SE	CV
Somatic cells	84.8 ± 4.1a	4.8	3.1 ± 0.2a	3.5	11.3 ± 0.4a	3.8
Premeiosis	57.7 ± 2.3b	3.9	30.7 ± 1.2b	3.8	14.1 ± 4.0b	0.3
Leptotene	61.1 ± 1.7bc	2.7	23.6 ± 1.4c	6.0	16.6 ± 0.8c	5.0
Zygotene	63.4 ± 1.4cd	2.2	18.9 ± 1.0d	5.3	18.6 ± 0.4d	2.3
Pachytene	63.6 ± 0.2cd	0.3	18.5 ± 0.8de	4.5	21.0 ± 1.0e	5.0
Diplotene	66.8 ± 0.2de	0.3	16.7 ± 0.4de	2.6	20.6 ± 1.3e	6.1
Diakinesis	69.2 ± 0.2ef	0.4	15.5 ± 1.2de	7.7	21.3 ± 0.6e	3.1
Metaphase I	72.8 ± 0.3f	0.4	14.5 ± 0.6e	4.4	21.8 ± 1.5e	6.9

Table 2: Flow cytometric determination of the percentage distributions of nuclei from wheat anthers in each phase of the cell cycle during meiosis in the presence of the *Ph1* locus. G0/G1 and G2/M values correspond to un-replicated and post-replicated cells, respectively. S phase values correspond to cells under active replication. Values are given as a mean of 9 measurements, standard error of the mean (SE) and coefficient of variation (CV). The same letter indicates no differences among treatments (stages of meiosis) within the same cell cycle phase (G0/G1, G2/M and S) at $P<0.05$.

	G0/G1 phase		S phase		G2/M phase	
	Mean ± SE	CV	Mean ± SE	CV	Mean ± SE	CV
Somatic cells	82.8 ± 3.1a	3.7	2.6 ± 0.6a	3.9	10.0 ± 0.6a	5.8
Premeiosis	57.1 ± 0.2b	0.4	30.5 ± 0.9b	2.8	12.4 ± 0.6b	5.0
Leptotene	62.6 ± 0.7c	1.1	24.4 ± 1.1c	7.9	17.3 ± 0.2c	4.1
Zygotene	63.7 ± 0.1cd	0.4	19.4 ± 0.5d	2.5	23.2 ± 0.5d	2.2
Pachytene	64.5 ± 0.4d	1.5	13.9 ± 0.9e	6.5	23.6 ± 1.4d	5.9
Diplotene	66.6 ± 0.3e	2.5	13.5 ± 0.7f	4.3	23.7 ± 0.3d	1.0
Diakinesis	68.3 ± 0.6f	1.9	13.5 ± 0.1f	0.3	23.3 ± 1.3d	5.5
Metaphase I	69.1 ± 0.6g	0.9	11.2 ± 0.7g	6.1	23.4 ± 1.5d	2.5

Table 3: Flow cytometric determination of the percentage distributions of nuclei from wheat anthers in each phase of the cell cycle during meiosis in the absence of the *Ph1* locus. G0/G1 and G2/M values correspond to un-replicated and post-replicated cells, respectively. S phase values correspond to cells under active replication. Values are given as a mean of 9 measurements, standard error of the mean (SE) and coefficient of variation (CV). The same letter indicates no differences among treatments (stages of meiosis) within the same cell cycle phase (G0/G1, G2/M and S) at $P<0.05$.

Dynamics of replication during early meiosis in hexaploid wheat

Flow cytometric analysis was carried out in wheat anthers to establish the temporal sequence of replication during early meiosis in wheat. To correctly stage the meiocytes during early meiosis, *in situ* hybridization was carried out to allow the visualization of chromosome dynamics by labelling centromeres and telomeres (Figure 1a). At the onset of meiosis most of the cells were located in G0/G1 phase (2C DNA content), which mostly corresponded to the somatic cells surrounding the meiocytes in the anther (Figure 1). The number of cells in G0/G1 phase slightly increased as meiosis progressed (Figure 1, Table 2), as a consequence of the anther cells multiplication to enlarge the anther size.

Interestingly enough, the number of cells under active replication in wheat anthers in premeiosis was much higher and significantly different than in the somatic control (30.7 $\pm$ 1.2 and 3.1 $\pm$ 0.2, respectively) (Table 2). In fact, the level of replication in wheat anthers in premeiosis was almost ten times higher than in the somatic tissue which reveals that replication is occurring during premeiosis in wheat. In addition, replicating cells (S value) were also detected in leptotene, zygotene and pachytene (Figure 1, Table 2). The replication values decreased from premeiosis (30.7 $\pm$ 1.2) to pachytene (18.5 $\pm$ 0.8). Then, replication remained constant from pachytene to diakinesis, but higher (15.5 $\pm$ 1.2) than the replication value obtained for the somatic control (3.1 $\pm$ 0.2). These results suggested that residual synthesis of DNA occurred in wheat after pachytene, when chromosomes are already associated in pairs.

According to these results, the number of cells already replicated in G2/M (4C DNA content) was higher and statistically different in premeiosis than in the somatic tissue (14.1 $\pm$ 4.0 and 11.3 $\pm$ 0.4 respectively), which correlates with replication in premeiosis in wheat anther. At the onset of meiosis the number of replicated cells increased from premeiosis (14.1 $\pm$ 4.0) up to pachytene (21.0 $\pm$ 1.0), being 1.9 times higher in pachytene than in the somatic control and

confirming that replication actively occurs during early meiosis in wheat (Figure 1, Table 2). The number of replicated cells from pachytene to metaphase I remained constant and almost double compared with the somatic control (21.8 $\pm$ 1.5 and 11.3 $\pm$ 0.4, respectively) (Table 2). These results confirm that replication occurs during early meiosis in wheat, as an increment in the number of replicated cells was clearly detected from premeiosis to pachytene. Hence, flow cytometry is an efficient tool to successfully detect and quantify replication during early meiosis in wheat, and shows that replication occurs actively from premeiosis until pachytene, when chromosomes are paired and telomeres are clustered at the bouquet.

Dynamics of replication during early meiosis in hexaploid wheat in the absence of the *Ph1* locus

Replication was also studied by flow cytometry in early meiosis in wheat in the absence of the *Ph1* locus, which affects replication and controls chromosome pairing during meiosis. Chromosome dynamics was tracked using *in situ* hybridization during early meiosis by labelling centromeres and telomeres to correctly stage meiosis (Figure 2a). As expected, most of the cells detected by flow cytometry corresponded to the G0/G1 cell cycle phase (2C peak) in all the stages analysed (Figure 2, Table 3). The number of cells in G0/G1 phase increased as long as meiosis progressed, consequence of the increment in the number of the somatic cells surrounding the meiocytes as the anther growths (Figure 2, Table 3).

Cells going under active replication (S phase) were also clearly detected in early meiosis in wheat in the absence of the *Ph1* locus using flow cytometry (Figure 2). In fact, the number of cells detected in replication was 11.7 times higher in premeiosis than in the somatic cell control in the absence of the *Ph1* locus (30.5 + 0.9 and 2.6 + 0.6, respectively) (Table 3). Replication was also detected in

leptotene and zygotene 9 and 7.5 times higher respectively than in the somatic control. Thus, replication decreased sharply from premeiosis until reaching zygotene (Table 3). The level of replication remained constant after zygotene but slightly higher than the somatic cell control (Table 3), suggesting that residual synthesis of DNA also occurred after zygotene in wheat anther in the absence of the *Ph1* locus. Therefore, active replication was detected by flow cytometry in wheat in the absence of the *Ph1* locus, with particularly high levels of replication in premeiosis and in early meiosis (leptotene and pachytene).

The number of cells in the G2/M phases of the cell cycle corresponding to replicated cells was significantly higher in premeiosis than in the somatic control (12.4 $\pm$ 0.6 and 10.0 $\pm$ 0.6, respectively). Moreover, the number of replicated cells did also increase during leptotene up to zygotene, where the level of replicated cells detected was almost double the number of replicated cells in premeiosis (23.2 $\pm$ 0.5 and 12.4 $\pm$ 0.6, respectively; Figure 2a, Table 2). Finally, the number of replicated cells remained constant from zygotene to metaphase I, being double the number of replicated cells detected either in premeiosis or in the somatic control (Table 3). Thus, these flow cytometric results clearly confirm that replication occurs during early meiosis in wheat in the absence of the *Ph1* locus, particularly in leptotene and zygotene, and can be monitored and quantified at each stage of meiosis.

Analysis of the effect of the *Ph1* locus on replication during meiosis in wheat

The effect of the *Ph1* locus on replication during early meiosis was analysed by flow cytometry. The amount of DNA was measured and compared for each meiotic stage in the presence and in the absence of the *Ph1* locus (Table 4). No differences were found in the number of unreplicated cells (G0/G1 phase) between wheat lines (*Ph1+*, *Ph1-*) during either premeiosis or any stage of prophase I (Figure 3a, Table 4). The only significance differences were found in

metaphase I between unreplicated cells of wheat lines in the presence and in the absence of the *Ph1* locus (72.8 ± 0.3 and 69.1 ± 0.6, respectively). These differences may be due to the differences in mature anther size in relation to the presence and absence of the *Ph1* locus, given that anthers in the *ph1b* mutant are slightly smaller than anthers in the presence of the *Ph1* locus.

In contrast, differences in replication during early meiosis in wheat have been revealed by flow cytometric analysis in the presence and in the absence of the *Ph1* locus. Our results showed that at the onset of meiosis replication occurred similarly in both wheat lines with no statistical differences in the amount of DNA either in the presence or in the absence of the *Ph1* locus (Figure 3b, Table 4). Moreover, no significant differences were detected for the S value in early prophase (leptotene and zygotene) between both wheat lines (*Ph1*+ and *Ph1*-) (Figure 3b, Table 4). However, differences in the level of replication were observed in pachytene in wheat in the presence and absence of the *Ph1* locus. Thus, in the presence of the *Ph1* locus replication is still occurring in pachytene (18.5 ± 0.8) meanwhile in the absence of the *Ph1* locus replication had already decreased and reached the basal level (13.0 ± 0.9; Table 4). Therefore, active replication seemed to terminate earlier (zygotene) in wheat in the absence of the *Ph1* locus. After pachytene replication remained similar in both wheat lines (Figure 3b, Table 4).

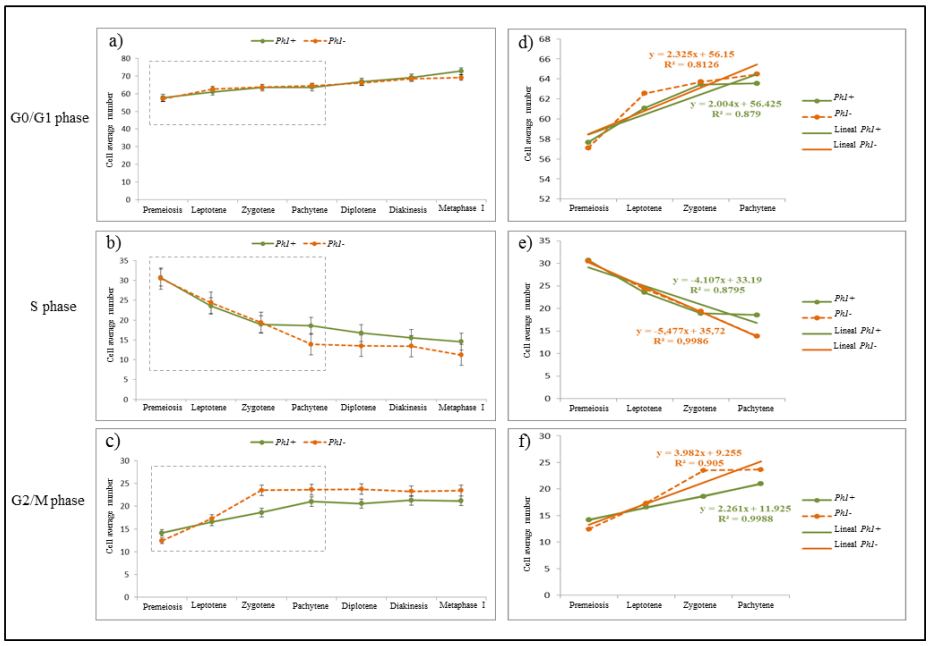

Figure 3. Comparison of the DNA content in wheat anthers in each phase of the cell cycle during the progression of meiosis, in the presence and in the absence of the *Ph1* locus. Each value represents the mean of 9 measurements at each meiotic stage. A. Percentage of cell numbers in each stage of the meiosis in G0/G1 phase of the cell cycle showing no differences in any stage due to the presence/absence of *Ph1* locus. B. Percentage of cell numbers in each stage of the meiosis in S phase. Differences were found in pachytene in the presence and in the absence of the *Ph1* locus. C. Percentage of cell numbers in each stage of the meiosis in G2/M phase. Differences were found in zygotene in the presence and in the absence of the *Ph1* locus. D. Regression line of the data points of the percentage of wheat nuclei represented in panel A. No differences were found for the slope of the line either in the presence or in the absence of the *Ph1* locus. E. Regression line of the data points of the percentage of wheat nuclei represented in panel B. The slope of the line for replication was higher (in absolute value) in the absence of the *Ph1* locus which means that the rate of replication is higher in its absence. F. Regression line of the data points of the percentage of wheat nuclei represented in panel C. The slope of the line was higher in the absence of the *Ph1* locus which implies that new replicated cells appear faster than in the presence of the *Ph1* locus.

	G0/G1 phase			S phase			G2/M phase		
	Mean ± SE	Mean ± SE	*P*	Mean ± SE	Mean ± SE	*P*	Mean ± SE	Mean ± SE	*P*
	Ph1+	***Ph1-***		***Ph1+***	***Ph1-***		***Ph1+***	***Ph1-***	
Premeiosis	57.7 ± 2.3a	57.1 ± 0.23a	0.89	30.7 ± 1.2a	30.5 ± 0.9a	0.89	14.1 ± 4.0a	12.4 ± 0.6a	0.12
Leptotene	61.1 ± 1.7a	62.6 ± 0.7a	0.44	23.6 ± 1.4a	24.4 ± 1.1a	0.57	16.6 ± 0.8a	17.3 ± 0.2a	0.09
Zygotene	63.4 ± 1.4a	63.7 ± 0.1a	0.88	18.9 ± 1.0a	19.4 ± 0.5a	0.06	18.6 ± 0.4a	23.2 ± 0.5b	0.02
Pachytene	63.6 ± 0.2a	64.5 ± 0.4a	0.11	18.5 ± 0.8a	13.0 ± 0.9b	0.03	21.0 ± 1.0a	23.6 ± 1.4a	0.28
Diplotene	66.8 ± 0.2a	66.1 ± 0.3a	0.11	16.7 ± 0.4a	13.5 ± 0.7a	0.18	20.6 ± 1.3a	23.7 ± 0.3a	0.07
Diakinesis	69.2 ± 0.2a	68.3 ± 0.6a	0.36	15.5 ± 1.2a	13.4 ± 0.1a	0.23	21.3 ± 0.6a	23.3 ± 1.3a	0.37
Metaphase I	72.8 ± 0.3a	69.1 ± 0.6b	0.02	14.5 ± 0.6a	11.2 ± 0.7a	0.10	21.2 ± 1.5a	23.4 ± 1.5a	0.43

Table 4: Comparison of the percentage of nuclei from wheat anthers in each phase of the cell cycle during early meiosis in the presence and in the absence of the *Ph1* locus (*Ph1+* and *Ph1-*, respectively). G0/G1 and G2/M values correspond to un-replicated and post-replicated cells, respectively. S phase values correspond to cells under active replication. Values are given as a mean of 9 measurements and standard error of the mean (SE). The same letter indicates no differences among treatments (*Ph1+* or *Ph1-*) within the same cell cycle phase (G0/G1, G2/M and S) in each stage of meiosis at P<0.05. Differences in replication due to the presence of the *Ph1* locus were only found in pachytene, as replication is still occurring in the presence of the *Ph1* at this stage of meiosis but has finished in its absence.

These data also suggest that there is no significant differences for the G2/M value between wheat lines in the presence and in the absence of the *Ph1* locus in any meiotic stage but in zygotene (Figure 3c, Table 4). The maximum number of replicated cells was reached in zygotene in the absence of the *Ph1* locus meanwhile the number of replicated cells did still increase up to pachytene in the presence of the *Ph1* locus, when the maximum value for the G2/M was reached (Table 4). Therefore, our results indicate that replication is occurring during early meiosis in wheat either in the presence or in the absence of the *Ph1* locus, although differences in the progression of replication have been detected. Our observations suggested that replication timing is affected by the *Ph1* locus as replication finished earlier (zygotene) in the absence of the *Ph1* locus. Moreover, the gradient of the line of the cells number in G0/G1, G2/M and S phases was calculated during early meiosis until pachytene, as no significant differences were found between both lines after this meiotic stage at any time, either in the presence or in the absence of the *Ph1* locus (Figure 3d-f). Results revealed that there are no statistical differences for the slope at the G0/G1 phase due to the presence of the *Ph1* as would be expected. In contrast, differences in the slope of the lines in both the S and the G2/M phases have been observed in the presence and in the absence of the *Ph1* locus. Moreover, the gradient of the line for the S phase was steeper (1.33 times) in the absence of the *Ph1* locus than in its presence, showing that the replication rate is higher in the absence of the *Ph1* locus. As expected, the gradient of the line for the G2/M phase was also higher (1.76 times) in the absence of the *Ph1* than in its presence, indicating that the rate of the increment in the number of replicated cells is higher in the absence of the *Ph1* locus. All these results suggest that replication timing is affected by the presence of the *Ph1* locus, in particular the rate of replication during early meiosis in wheat. The replication rate during meiosis is lower in the presence of the *Ph1* locus. Consequently replication during meiosis in wheat lasts longer in the presence of the *Ph1* locus.

Discussion

The cell cycle is a much studied process due to its importance in plant growth and development. The significance of replication during the cell cycle is critical to ensure proper chromosome association, recombination and segregation in meiosis, which is directly related to viability of gametes and therefore to fertility. This paper presents a simple and robust method for the determination of the synthesis of DNA during early meiosis by means of flow cytometric measurements in nuclei released from fixed wheat anthers. The synthesis of DNA has been studied using different deoxynucleosides, such as [^{3}H] thymidine, 5-bromo-2'-deoxyuridine (BrdU) or 5-ethynyl-2'-deoxyuridine (EdU), which is highly sensitive (Salic and Mitchison, 2008). Nevertheless, genome size determination, which can be correlated with the synthesis of DNA, must be carried out with a DNA intercalation dye that allows total DNA staining, such as ethidium bromide, propidium iodide, or DAPI (in this work) which provides DNA content histograms with high resolution, uses readily available excitation wavelengths and does not require RNAse treatment of samples. Furthermore, flow cytometry is cheaper than other methods for analysing DNA replication and has already allowed a rapid and accurate analysis of large populations of cells (Dolezel, 1998). In fact, flow cytometry has already been applied in plants to determine the nuclear replication stages in seeds from *Lactuca sativa* L., *Solanum melongena* L., and *Lycopersicon esculentatum* Mill., among other species (Bino *et al.*, 1992; Bino *et al.*, 1993). Fixation of the samples is also often convenient in experiments involving multiple and complex samples. However, fixed nuclear preparations often display wider G1 and G2 peaks in flow cytometric histograms (Kotogány *et al.*, 2010). Nevertheless, although in this work samples were fixed, CV values were below 5% in most of the cases and always lower than the 8%. However, and to the best of our knowledge, it is the first time that this approach is used to quantify replication during meiosis in a crop such as wheat. Replication has

been studied in early meiosis in wheat-rye hybrids through the incorporation of EdU (Greer *et al.*, 2012). Using flow cytometry we established in this work that DNA synthesis is occurring in early stages of meiosis in common wheat, and quantified the rate of replication during meiosis and the stages of meiosis in which replication occurs. Moreover, using this methodology we have also been able to study the role of the presence of the *Ph1* locus, which controls chromosome pairing (Riley and Chapman, 1958), on DNA replication during meiosis in wheat. Thus, we observed that chromosome pairing was initiated before the completion of replication, as telomeres started to associate to form a bouquet when replication was still occurring in both wheat lines, either in the presence or in the absence of the *Ph1* locus, similarly to the observations of (Greer *et al.*, 2012). But differences in timing of replication during meiosis were found in the presence and in the absence of the wheat *Ph1* locus. Thus replication last longer (until pachytene) during early meiosis in the presence of the *Ph1* locus, or in other words, replication finished earlier (zygotene) when the *Ph1* locus was absent. In fact, our analysis of the slope of the lines at early meiosis indicates that the rate of replication during meiosis is higher in the absence of the *Ph1* locus. Due to the fact that the *Ph1* locus is similar to Cdk2 (Griffiths *et al.*, 2006) and Cdk2 affects replication (Thomson *et al.*, 2010), our results confirm one the hypotheses proposed previously (Greer *et al.*, 2012) by studying wheat-rye hybrids that in the absence of the *Ph1* locus, in that the activation of origins of replication might be increased and consequently the rate of replication. Residual replication was also detected in wheat anthers at later meiosis stages, after pachytene and zygotene in wheat in the presence and in the absence of the *Ph1* locus, respectively, when chromosomes are associated in pairs. Replication at this stage of meiosis corresponds not only to heterochromatin regions which replicate later in meiosis (Greer *et al.*, 2012) but also to cell division and therefore residual replication of the somatic surrounding cells.

The structure of chromatin has been shown determinant for the initiation of replication (Gilbert, 2001). Moreover, homologous chromosomes usually replicate synchronously although there are some exceptions (Gilbert, 2002). In addition, replication of the chromatin has been shown temporary ordered in barley (Jasencakova *et al.*, 2001). Our results suggested that sequential replication might be carried out in wheat in the presence of the *Ph1* locus to facilitate correct chromosome associations, and consequently replication takes longer. This could also suggest that only homologous chromosomes are replicating at the same, which could be associated with similar homologous chromosome conformation at early meiosis in wheat, as it has previously described (Prieto *et al.*, 2004a; Colas *et al.*, 2008). Finally, our results support previous findings on replication during early meiosis in wheat-rye hybrids in the presence and in the absence of the *Ph1* locus, explained as an increment in the activation of origins and hence the rate of replication of disperse chromatin in wheat-rye hybrids in the absence of the *Ph1* locus (Greer *et al.*, 2012).

In summary, flow cytometry has been revealed as a suitable tool to detect and quantify DNA replication during early meiosis in wheat. Replication was detected in wheat during premeiosis and early meiosis until the stage of pachytene, when chromosomes are associated in pairs to further recombine and correctly segregate in the gametes. Moreover, flow cytometric results suggested that the *Ph1* locus is affecting the rate of replication during early meiosis in wheat, being lower in the presence of the *Ph1* locus and consequently, replication during early meiosis lasts longer and finishes later than in the absence of the *Ph1* locus. The biological significance of replication at early meiosis and the effect of the *Ph1* locus in replication suggest a solid connection between DNA replication and chromosome associations at the onset of meiosis

in a polyploid like wheat. Further studies are needed to build upon these results for unravelling the underlying molecular mechanisms.

Chapter III

The use of the ph1b *mutant to induce recombination between the chromosomes of wheat and barley*

Under review in:

Rey MD, Calderón MC, Prieto P (2015) The use of the *ph1b* mutant to induce recombination between the chromosomes of wheat and barley. Frontiers in Plants Science.

Abstract

Intensive breeding has led to a narrowing in the genetic base of our major crops. In wheat, access to the extensive gene pool residing in its many and varied relatives (some cultivated, others wild) is hampered by the block on recombination imposed by the *Ph1 (Pairing homologous 1)* gene. Here, the *ph1b* mutant has been exploited to induced allosyndesis between wheat chromosomes and those of both *Hordeum vulgare* (cultivated barley) and *H. chilense* (a wild barley). A number of single chromosome *Hordeum* sp. substitution and addition lines in wheat were crossed and backcrossed to the *ph1b* mutant to produce plants in which pairing between the wheat and the non-wheat chromosomes was not suppressed by the presence of *Ph1*. Genomic *in situ* hybridization was applied to almost 500 BC_1F_2 progeny as a screen for allosyndetic recombinants. Chromosome rearrangements were detected involving the *H. chilense* chromosomes 4$\mathbf{H}^{ch}$, 5$\mathbf{H}^{ch}$, 6$\mathbf{H}^{ch}$ and 7$\mathbf{H}^{ch}$ and the *H. vulgare* chromosomes 4$\mathbf{H}^{v}$, 6$\mathbf{H}^{v}$ and 7$\mathbf{H}^{v}$. Two of these were clearly the product of recombination event involving chromosome 4$\mathbf{H}^{ch}$ and a wheat chromosome.

Keywords: *Triticum*, *Hordeum* substitution and addition lines, *Ph1* locus, wheat breeding, recombination, meiosis

Introduction

Bread wheat (*Triticum aestivum*) is one of the most important food crops of the world, and continuous improvement in its productivity will be required to keep pace with global population growth. The genetic base of the species is rather narrow, as its speciation was very recent (Riehl *et al.*, 2013; Salamini *et al.*, 2002). However, a large number of sexually compatible species (some wild and some cultivated) are known, and these represent a much needed reservoir of potentially exploitable genetic variation.

The genome of an interspecific or (intergeneric) hybrid combines the haploid complements of each of its sexual parents. Even though their genomes are closely related to one another, in most cases, the chromosomes of wheat and those of its relatives fail to pair with one another and thus allosyndetic recombination is rare. The failure of homoeologues (chromosomes from related genomes but not completely homologous) to interact at meiosis is ensured by the wild type allele at the *Ph1* locus (Okamoto, 1957; Riley and Chapman, 1958; Sears and Okamoto, 1958; Sears, 1977), the impact of which is to impose diploid-like chromosome behaviour during meiosis in any wild type wheat, even though the constituent sub-genomes of this hexaploid species are known to be very closely related to one another. Deletion of the *Ph1* locus, which comprises a cluster of Cdk2-like genes (Griffith *et al.*, 2006; Al-kaff *et al.*, 2008; Yousafzai *et al.*, 2010), allows homoeologues to pair relatively freely with one another, a situation which has been exploited for introgression purposes through the use of the *ph1b* mutant (Sears, 1977, 1981, 1982; Riley *et al.*, 1968; Khan, 1999; Lukaszewski, 2000; Qi *et al.*, 2008; Liu *et al.*, 2011; Zhao *et al.*, 2013).

Hordeum chilense, a species which is readily crossable with wheat, is a diploid relative of cultivated barley. It has been identified as a potential donor to wheat for a number of traits of agronomic interest (Martín *et al.*, 1998, 2000). The

bread wheat x *H. chilense* hybrid has been the source of a collection of single (*Hordeum*) chromosome addition lines and chromosome substitution lines in a bread wheat genetic background (Miller *et al.,* 1982), and similar cytogenetic stocks have been developed involving the cultivated barley (*Hordeum vulgare*) chromosomes (Islam *et al.,* 1978, 1981). The self-fertile amphidiploid ×*Tritordeum* represents the product of chromosome doubling of the hybrid *T. turgidum* x *H. chilense* (Martín and Sanchez-Monge-Laguna, 1982). The presence of *Ph1* maintains the integrity of *Hordeum* sp. chromosome(s) in all of this germplasm, meaning that the introgression of favourable non-wheat genes is inevitably accompanied by the inheritance of a large number of unwanted ones. The experience with introgression into wheat from other related species suggests that this linkage drag can best be overcome by employing a *ph1b*-based strategy. Here, in this chapter we describe progress made with an introgression programme using the *ph1b* mutant to induce chromosome pairing and recombination between the chromosomes of *H. chilense* or *H. vulgare* and those of wheat.

Materials and methods

Plant materials

Table 1 lists the various *H. chilense* substitution lines and *H. chilense* and *H. vulgare* addition lines (Miller *et al.,* 1982; Islam *et al.,* 1978, 1981) used as the female parent in crosses with the *ph1b* mutant (Sears, 1977). All the lines used in this chapter were kindly supplied by Dr. Steve Reader (JIC, Norwich, UK). Grains were germinated on wet filter paper in the dark for five days at 4ºC, followed by a period of 24 h at 25ºC. Emerging seedling roots were excised, incubated for 4 h in 0.05% w/v colchicine at 25ºC, fixed in Carnoy's solution (three parts 100% ethanol plus one part glacial acetic acid) and finally stored at 4ºC for at least one month. The plants were subsequently raised in a greenhouse held at 26ºC during the day and 22ºC during the night (16 h photoperiod).

Immature spikes were preserved in Carnoy's solution and used to characterize chromosome pairing at meiosis metaphase I.

Initial parental lines				**Descendence**		
Wheat line (female), nomenclature and number of plants used			**CS*ph1ph1* (male)**	**F1**	**BC1F1**	**BC1F2**
(4**B**)4**H**^{ch} disomic substitution line	CS(4**B**)4**H**^{ch}	5	3	15	17	30
(4**D**)4**H**^{ch} disomic substitution line	CS(4**D**)4**H**^{ch}	5	3	11	15	48
(5**A**)5**H**^{ch} disomic substitution line	CS(5**A**)5**H**^{ch}	5	3	16	22	12
(5**B**)5**H**^{ch} disomic substitution line	CS(5**B**)5**H**^{ch}	5	3	15	48	30
(5**D**)5**H**^{ch} disomic substitution line	CS(5**D**)5**H**^{ch}	5	3	11	22	20
(7**A**)7**H**^{ch} disomic substitution line	CS(7**A**)7**H**^{ch}	5	3	21	47	77
(7**B**)7**H**^{ch} disomic substitution line	CS(7**B**)7**H**^{ch}	5	3	19	59	64
(7**D**)7**H**^{ch} disomic substitution line	CS(7**D**)7**H**^{ch}	5	3	19	36	40
5**H**^{ch} disomic addition line	5**H**^{ch} addition	5	3	5	27	-
6**H**^{ch} disomic addition line	6**H**^{ch} addition	5	3	29	35	20
7**H**^{ch} disomic addition line	7**H**^{ch} addition	5	3	16	21	25
Total of wheat-*H. chilense* plants		**55**	**33**	**177**	**349**	**366**
2**H**^{v} disomic addition line	2**H**^{v} addition	5	3	11	20	-
4**H**^{v} disomic addition line	4**H**^{v} addition	5	3	15	52	46
6**H**^{v} disomic addition line	6**H**^{v} addition	5	3	23	33	23
7H^{v} disomic addition line	7**H**^{v} addition	5	3	21	28	38
Total of wheat-*H.vulgare* plants		**20**	**12**	**70**	**133**	**107**
Total		**75**	**45**	**218**	**482**	**473**

Table 1. Plants used for crosses made to engineer individuals carrying a *Hordeum* sp. chromosome in a *ph1b* mutant background. CS: wheat cv. Chinese Spring, **H**^{ch}: *H. chilense*. **H**^{v}: *H. vulgare*

DNA marker characterization

Genomic DNA was extracted from frozen seedling leaves following Murray and Thomson, (1980), as modified by Hernández *et al.*, (2001). The absence of *Ph1* was verified using a PCR assay described by Wang *et al.,* (2002). Each 30 µL

PCR contained 1x PCR buffer with $MgCl_2$ (Bioline USA, Taunton, MA, USA), 0.25 mM dNTP, 0.17 µM primers, 0.02 U/µL Taq DNA polymerase (Bioline USA) and 20 ng template. The reaction was first denatured (94ºC/5 min), then subjected to 35 cycles of 94ºC/60 s, 51ºC/60 s, 72ºC/60 s, followed by a final extension (72ºC/7 min). The PCR products were electrophoretically separated through a 1% agarose gel and visualized by EtBr staining. The presence of each *Hordeum* sp. chromosome was based on PCR assays described by Hagras *et al.*, (2005) and Liu *et al.*, (1996), as detailed in Table 2. The composition of these PCRs was as above, while the amplification regime comprised an initial denaturing step (94ºC/5 min), followed by 35 cycles of 94ºC/15 s, 50-65ºC (primer dependent, see Table 2)/30 s, 72ºC/60 s, and completed by a final extension (72ºC/6 min). The amplicons were separated as described above.

Cytogenetic analysis

Chromosome spreads were prepared from both pollen mother cells (PMCs) at meiotic metaphase I and from root tip cells. The material was macerated in a drop of 45% glacial acetic acid, squashed under a cover slip and dipped in liquid nitrogen in order to remove the cover slip. The preparations were then air-dried and either processed directly for *in situ* hybridization, or stored at 4ºC until required. The probe used for genomic *in situ* hybridization was genomic DNA extracted from *H. chilense* (or *H. vulgare*) seedling leaves. The DNA was labelled with either biotin-11-dUTP (*H. vulgare*) or digoxigenin-11-dUTP (*H. chilense*) (both from Roche Corporate, Basel, Switzerland) by nick-translation. The *in situ* hybridization protocol followed that described by Prieto *et al.*, (2004). The GAA-satellite sequence (Pedersen *et al.*, 1996) and the pAs1 probe (Rayburn and Gill, 1986) were used to identify chromosomes involved in homoeologous pairing, chromosomal translocations or chromosomal rearrangements. The GAA-satellite sequence identifies all the **A** and **B** wheat chromosomes (Pedersen and Langridge, 1997), whereas the pAs1 identifies the

D wheat and the *H. chilense* chromosomes (Cabrera *et al.*, 1995). The GAA-satellite sequence and the pAs1 probes were also labelled by nick translation with biotin-11-dUTP and digoxigenin-11-dUTP, respectively. Biotin- or digoxigenin-labelled DNA were detected using respectively streptavidin-Cy3 (Sigma, St. Louis, MO, USA) and antidigoxigenin-FITC (Roche Applied Science, Indianapolis, IN, USA). After counter-staining with DAPI (4', 6-diamidino-2-phenylindole), the preparations were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA). Hybridization signals were visualized using a Nikon Eclipse 80i epifluorescence microscopy, and the images captured with a CCD camera (Nikon Instruments Europe BV, Amstelveen, The Netherlands).

Statistical analysis

Statistical analyses were performed using the STATISTIX v9.0 software (Analytical Software, Tallahassee, FL, USA). Wilcoxon (or U of Mann-Whitney) test was used to determine the statistical significance of differences between means.

Results

Converting the substitution and addition lines into a *ph1b* mutant background

The crossing scheme used is illustrated in Figure 1, and the details of the crossing outcomes through to the BC_1F_1 generation are given in Table 1. The F_1 hybrid progeny were genotyped to ensure that they had retained the expected *Hordeum* sp. chromosome (Table 2, Figure 2a), then crossed again to the *ph1b* mutant in order to establish individuals in which the *Hordeum* sp. chromosome was now present in a *ph1bph1b* background. Zygosity at the *Ph1* locus was predicted using a PCR assay (Figure 2b). The meiotic behaviour of the

selections was characterized by GISH analysis of metaphase I in PMCs, and the plants were allowed to self-pollinate.

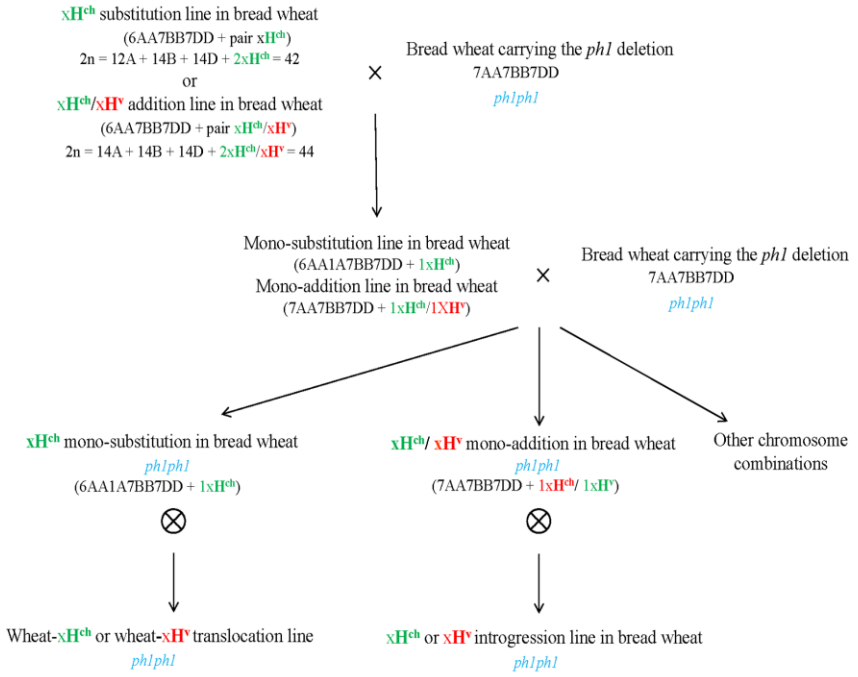

Figure 1. Development of *Hordeum* sp. introgression lines in hexaploid wheat in the *ph1b* mutant background. Crosses between a *Hordeum* sp. substitution or addition line in bread wheat cv. Chinese Spring (2n = 6x = 42) and the *ph1b* mutant in hexaploid wheat were developed and backcrossed to the *ph1b* mutant to obtain *Hordeum* sp. introgressions in the absence of the *Ph1* locus. Screening and characterization of chromosome complements were carried out by multicolor *in situ* hybridization and molecular markers analysis.

Marker name	Sequence of primers (5'→3')	*Hordeum* chromosome	Annealing temperature (ºC)
BAWU759-F	TCGACATCTCTCCCATTTCCC	2**H**-S	50
BAWU759-R	AACCAGATATGGATGCCAGG	2**H**-S	50
HVCSG-F*	CACTTGCCTACCTCGATATAGTTTGC	2**H**^{v}-L	50
HVCSG-R*	GTGGATTCCATGCATGCAATATGTGG	2**H**^{v}-L	50
BAWU303-F	AATGTGCCTCCACAGGGTAG	4**H**-S	55
BAWU303-R	GATACTGAGTGGAAAGCGGC	4**H**-S	55
BAWU808-F	TGCCCCCAAACTTTATATGC	4**H**-L	55
BAWU808-R	GAGGGTCTTCCTGTTGTGGA	4**H**-L	55
BAWU131-F	GAACGCCAGCCAAATTGTAT	5**H**-S	60
BAWU131-R	ACCATTTTGATCCTTCTGCG	5**H**-S	60
BAWU782-F	CAACTTGGACAACACAACGC	5**H**-L	60
BAWU782-R	CTTGTGCATGCGCAGAGTAT	5**H**-L	60
BAWU94-F	TTTCAAGCAGAGCTGCAAAG	6**H**-S	55
BAWU94-R	GCTTGCTGAGCGCTTTCTAC	6**H**-S	55
BAWU107-F	CGCCTATTTCTGAGCTCCTG	6**H**-L	55
BAWU107-R	CGAGTATGGGAGTGGCAGTT	6**H**-L	55
BAWU763-F	AGAACCGAGATGAGGAATGTG	7**H**-S	58
BAWU763-R	AGTCTCTTCGCGGAATCAAG	7**H**-S	58
BAWU550-F	ATGCCACCATTTACAAAGCC	7**H**-L	50
BAWU550-R	TTTCTGGGTCCTGATCCTTG	7**H**-L	50

Table 2. DNA-based markers employed as genotypic assays for the presence of a *Hordeum* sp. chromosome. F: Forward primer, R: reverse primer. **H**^{v}: *H. vulgare*, **H**: *H. chilense* and *H. vulgare*

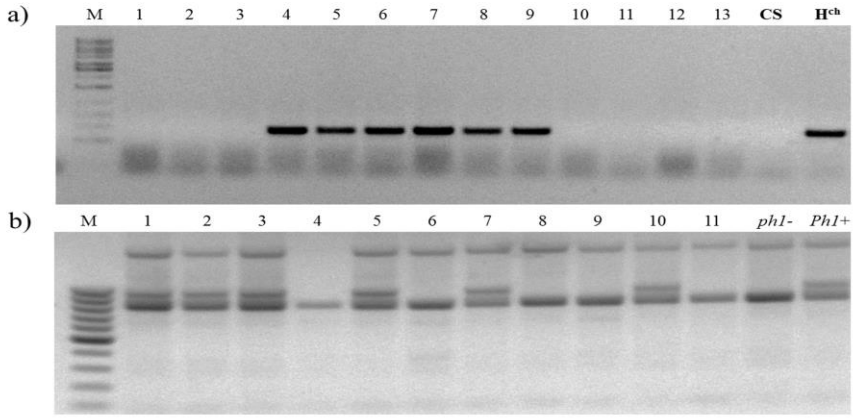

Figure 2. Genotypic assays for the presence of *Ph1* and a *Hordeum* sp. chromosome. A. The presence of chromosomes 4**H**^{ch} (lanes #4-#9) is marked by the successful amplification of the BAWU303 EST fragment. B. The absence of *Ph1* is marked by the loss of the ABC_{920} SCAR marker (individuals #4, #6, #8, #9 and #11) M: size marker; *ph1b*: the parental *ph1b* mutant, *Ph1*: wild type wheat. CS: *Triticum aestivum* cv. Chinese Spring*;* **H**^{ch}: *H. chilense*.

Allosyndetic pairing in BC_1F_1 selections lacking *Ph1*

Meiosis was characterized in 63 BC_1F_1 segregants carrying a *Hordeum* chromosome in the absence of the *Ph1* and compared to those carrying the *Hordeum* chromosome in its presence (Table 3). No wheat/*Hordeum* chromosome pairing occurred in plants of genotype *Ph1Ph1* (Table 3, Figure 3a, d). In contrast, in the absence of the *Ph1* locus, although the *Hordeum* chromosomes remained unpaired in most metaphase I PMCs (Figure 3 b, e), pairing was observed in 1.77% of the PMCs in *H. chilense* (Table 3, Figure 3c). The equivalent frequency with respect to *H. vulgare* chromosomes was 1.84% (Table 3, Figure 3f). The frequency of plants displaying wheat/*Hordeum* chromosome associations was lower in *H. chilense* than in *H. vulgare* (45.23% and 61.90%, respectively), although variability depending on the specific *Hordeum* sp. chromosome introgressed was found. Most of the associations between a *Hordeum* and a wheat chromosome involved the formation of a rod

bivalent harboring a single sub-terminal chiasma (Figure 4a-a''), although in some cases the chiasma occurred more proximally (Figure 4b-b''). In a few PMCs, the *Hordeum* sp. chromosome formed part of a multivalent (Figure 4c-c''), reflecting the genomic re-arrangement of the wheat genome induced by successive meiosis during the generations of selfing used to maintain the *ph1b* mutant stock. Wilcoxon test showed that the frequency of allosyndesis was not *Hordeum* sp. chromosome specific, since there was no significant difference in pairing frequency between either chromosomes 4$\mathbf{H^{ch}}$, 6$\mathbf{H^{ch}}$ and 7$\mathbf{H^{ch}}$ or between chromosomes 4$\mathbf{H^{v}}$, 6$\mathbf{H^{v}}$ and 7$\mathbf{H^{v}}$ (Table 4a). In addition, using the same statistical test, no significance differences were found when compared the effect of the genome (*H. chilense* or *H. vulgare*) for the same homoeologous group (p=0.39, 0.41 and 0.70 for chromosomes 4, 6 and 7, respectively; Table 4a). A statistical comparison of chromosome pairing frequency involving a *H. chilense* chromosome and each of its wheat homoeologues was also carried out and showed no evidence for any preferential pairing (Table 4b). GISH analysis at the tetrad stage identified three distinct situations: (1) two of the four tetrads carrying *H. chilense* chromatin, corresponding to the inheritance of a full *H. chilense* chromosome by reassortment; ii) three of the four tetrads carrying *H. chilense* chromatin, corresponding to the occurrence of a recombination event between the *H. chilense* chromosome and a wheat homoeologue; and iii) all four tetrads carrying *H. chilense* chromatin, which could correspond with two or more recombination events between *H. chilense* and wheat (Figure 5). Chromosome breaks or interspecific chromosome translocations cannot be discarded.

Wheat line	*Hordeum* sp. introgressed	Nº of plants analysed	Nº of plants showing wheat-*Hordeum* pairing	Frequency of wheat-*Hordeum* pairing (%)	Nº of PMCs scored	Nº of PMCs scored showing wheat-*Hordeum* pairing	Frequency of wheat-*Hordeum* pairing in PMCs (%)	*p value*
Ph1^{+}		5	0	0.00	206	0	**0.00**	p=0.000***
ph1-	*H. chilense*	42	19	45.23	2422	43	1.77	
	H. vulgare	21	13	61.90	1352	25	1.84	
	Total	63	32	53.56	3774	67	**1.80**	

Table 3. The frequency of allosyndesis involving a *Hordeum* and a wheat chromosome in either the presence (*Ph1*+) or absence (*ph1*-) of the *Ph1* locus.

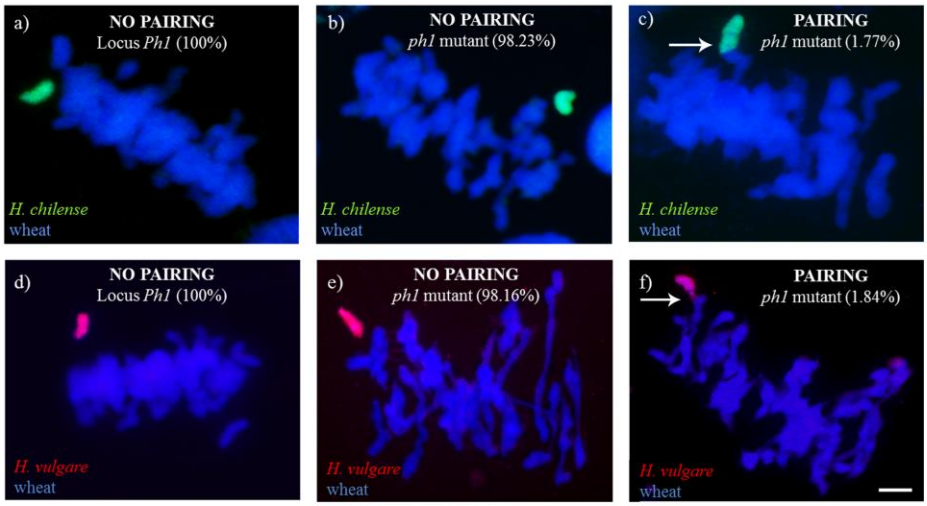

Figure 3. Chromosome pairing at meiotic metaphase I as determined by the allelic status at *Ph1*. In the presence of *Ph1*, the *Hordeum* sp. Chromosome (A. 7$\mathbf{H}^{ch}$, shown in green and D. 4$\mathbf{H}^{v}$, shown in red) remained unpaired. In a *ph1b* background, the *Hordeum* sp. chromosome (B. 5$\mathbf{H}^{ch}$, shown in green and E. 7$\mathbf{H}^{v}$, shown in red) remained as a univalent in most cells. Allosyndesis is induced by the absence of *Ph1* between a *Hordeum* sp. chromosome (C. 5$\mathbf{H}^{ch}$, shown in green and F. 7$\mathbf{H}^{v}$, shown in red), and a wheat chromosome. Arrows indicate bridges formed between *Hordeum* sp.-wheat homoeologues induced by the absence of the *Ph1* locus. Bar: 10µm.

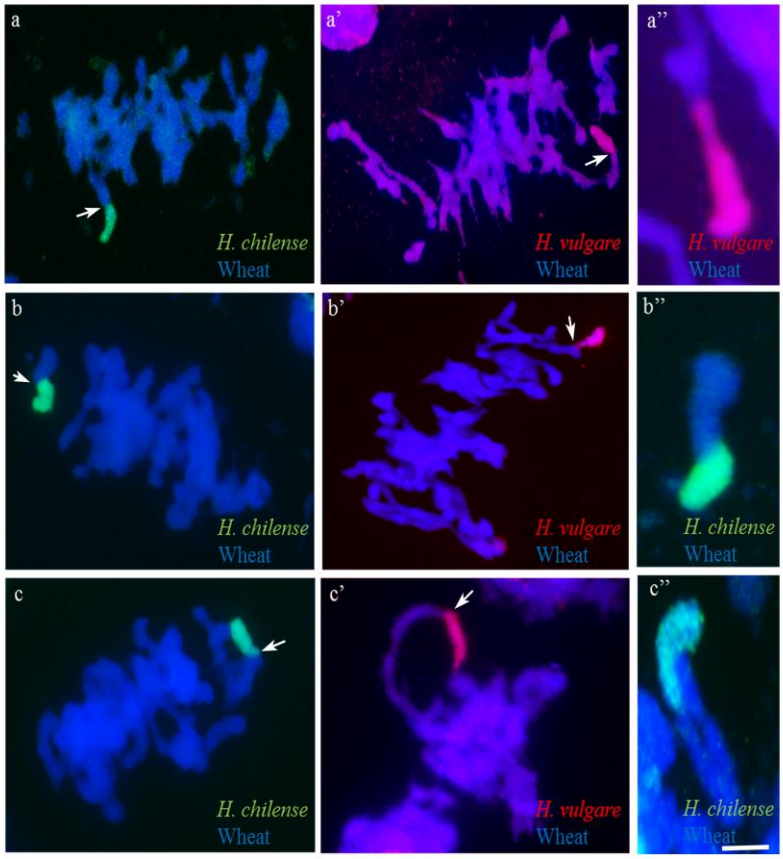

Figure 4. *Hordeum* sp./wheat chromosome pairing at meiotic metaphase I as detected by GISH. (A.-A.'') Rod bivalents with a sub-terminal chiasma. (B.-B.'') Rod bivalents with a more proximal chiasma. (C.-C.'') A *Hordeum* sp. chromosome involved in a multivalent. Bar: 10µm.

a)	Frequency of *Hordeum*-wheat pairing (%)			
Genome	**Chromosome 4**	**Chromosome 6**	**Chromosome 7**	***p* value**
H. chilense	1.59	1.65	1.83	0.63(p>0.05)
H. vulgare	1.24	2.78	0.86	0.75(p>0.05)
***p* value**	0.39 (p>0.05)	0.41 (p>0.05)	0.70 (p>0.05)	

b)	Frequency of *Hordeum*-wheat pairing (%)		
wheat homoeology group	**Chromosome $4H^{ch}$**	**Chromosome $5H^{ch}$**	**Chromosome $7H^{ch}$**
A	-	3.55	0.79
B	0.31	2.85	2.78
D	2.87	2.68	4.09
***p* value**	0.37 (p>0.05)	0.42 (p>0.05)	0.30 (p>0.05)

Table 4. A. The frequency of allosyndesis between individual *H. chilense* or *H. vulgare* chromosomes and those of wheat. B. The frequency of pairing between specific *Hordeum* chromosomes and each of their wheat homoeologues.

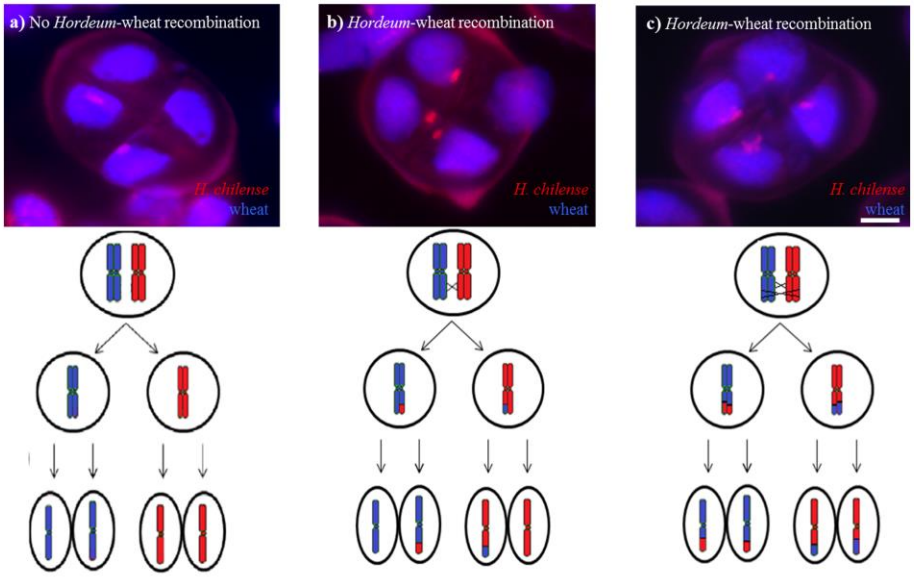

Figure 5. Tetrad analysis of the transmission of *H. chilense* chromosomes. Bar: 10µm.

Genetic evidence for *Hordeum* sp. introgression induced by the absence of *Ph1*

A total of 473 BC_1F_2 progeny were subjected GISH analysis to detect and characterize *Hordeum* sp. chromosome re-arrangements in the background of the *ph1b* mutant. About 60% of the progeny lacked any *Hordeum* sp. chromatin. The highest transmission rate of a *Hordeum* chromosome was observed among the progeny derived from the (4**B**) 4$\mathbf{H}^{\mathbf{ch}}$ substitution line. A number of mis-division products were observed among the progeny of *ph1b* selections, involving chromosomes 6$\mathbf{H}^{\mathbf{ch}}$, 7$\mathbf{H}^{\mathbf{ch}}$, 4$\mathbf{H}^{\mathbf{v}}$, 6$\mathbf{H}^{\mathbf{v}}$ and 7$\mathbf{H}^{\mathbf{v}}$ (Table 5, Figure 6). Overall, with respect to the *Hordeum* sp. chromosome, about 3% of the progeny were disomic and about 33% were monosomic. A total of 15 individuals harbored a Robertsonian translocation involving a *H. chilense* (chromosome 5$\mathbf{H}^{\mathbf{ch}}$: one plant, chromosome 7$\mathbf{H}^{\mathbf{ch}}$: 14 plants) and a wheat chromosome (Table

5, Figure 6). Two recombinants were identified, both involving chromosome 4**H**^{ch} (Table 5, Figure 6).

Wheat line	**Nº of plants**						
	complete chromosome			***Hordeum*-wheat**	**telosomic**	**small**	
	2 copies	**1 copy**	**0 copies**	**translocations**	**chromosome**	**introgression**	**Total**
CS(4B)4H^{ch}	2	13	15	0	0	0	30
CS(4D)4H^{ch}	0	15	31	0	0	**2 (4.2%)**	48
CS(5A)5H^{ch}	0	5	7	0	0	0	12
CS(5B)5H^{ch}	1	10	18	**1 (3.3%)**	0	0	30
CS(5D)5H^{ch}	0	5	15	0	0	0	20
CS(7A)7H^{ch}	2	32	37	**5 (6.3%)**	1	0	77
CS(7B)7H^{ch}	1	20	37	**4 (6.2%)**	2	0	64
CS(7D)7H^{ch}	0	11	26	**3 (7.5%)**	0	0	40
6H^{ch} addition	0	8	11	0	1	0	20
7H^{ch} addition	2	6	15	**2 (8%)**	0	0	25
4H^{v} addition	3	14	28	0	1	0	46
6H^{v} addition	2	9	11	0	1	0	23
7H^{v} addition	1	10	25	0	2	0	38
Total	14	158	276	15	8	2	**473**

Table 5. BC$_1$F$_2$ progeny retaining *H. chilense* or *H. vulgare* chromatin.

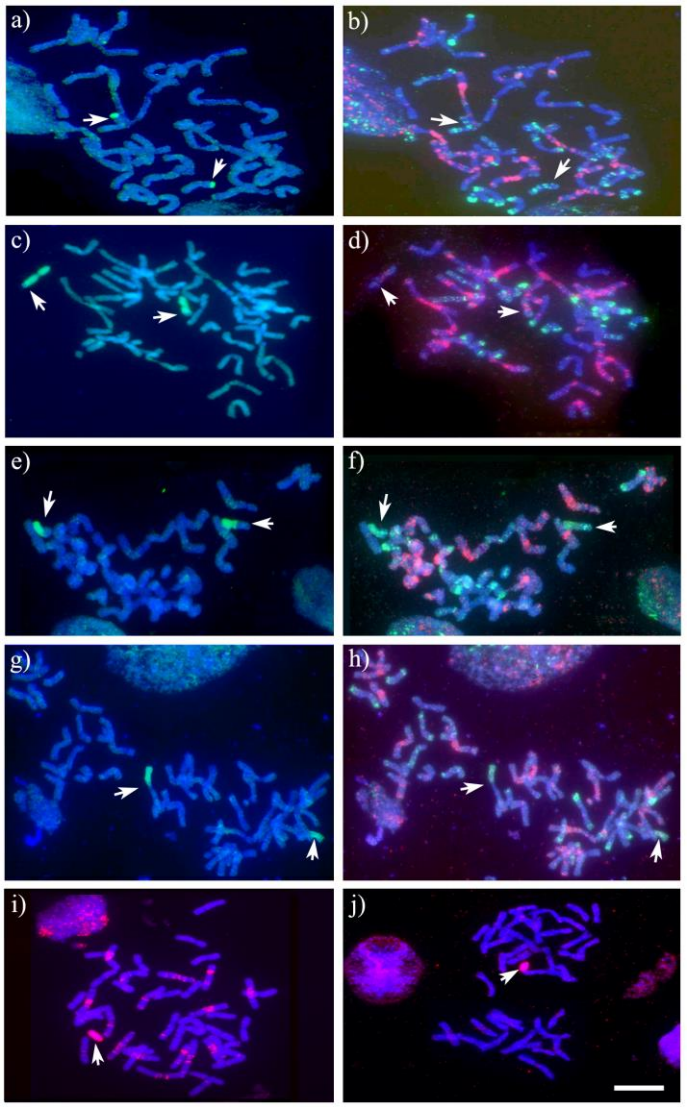

Figure 6. Various forms of introgression (arrowed) detected by GISH and FISH patterns in the BC_1F_2 progeny derived from the crosses *H. chilense* addition/substitution line x *ph1b* mutant*2. A. GISH and B. FISH pattern of chromosomes of a partial mitotic metaphase carrying two copies of a 4**D** chromosome with a distal 4$\mathbf{H^{ch}}$L segment. C. GISH and D. FISH pattern of a mitotic metaphase carrying two copies of a 4$\mathbf{H^{ch}}$ chromosome with a distal 4**D**L segment. E. GISH and F. FISH pattern of a mitotic metaphase carrying a 7$\mathbf{H^{ch}}$S-7**A**L Robertsonian translocation. G. GISH and H. FISH pattern of a mitotic metaphase carrying a 7**A**S-7$\mathbf{H^{ch}}$L Robertsonian translocation. I. GISH of a 4$\mathbf{H^{v}}$ monotelosomic line. J. GISH of a 6$\mathbf{H^{v}}$ monotelosomic line. Bar: 10µm.

Discussion

Interspecific hybridization retains its potential to widen the gene pool available to the wheat breeder. Combining *in situ* hybridization with DNA-based genotyping has eased the process considerably since the initial efforts which followed the recognition that recombination could be induced by the deletion of *Ph1* (Koebner and Shepherd, 1986; Qi *et al.*, 2007). An *in situ* hybridization-based screening strategy has previously been applied to characterize introgressions from both *H. chilense* and *H. vulgare*, resulting in the recognition of a number of wheat/*Hordeum* sp. translocations (Prieto *et al.*, 2001). Here, the intention was to exploit the abolition of strict homologue pairing induced by the absence of *Ph1* to generate, if possible, material where recombination had shortened the length of the introgressed segment. Chromosome 4$\mathbf{H^{ch}}$ is of particular interest as it harbors a gene (or possibly genes) encoding resistance against the fungal pathogen *Septoria tritici* (Rubiales *et al.*, 2000). Similarly, chromosome 7$\mathbf{H^{ch}}$ has been targeted for its positive effect on grain carotenoid content (Alvarez *et al.*, 1998), and chromosome 5$\mathbf{H^{ch}}$ for its contribution to enhancing salinity tolerance (Forster *et al.*, 1990). Although inter-chromosome translocations are known to occur spontaneously (Mettin *et al.*, 1973; Zeller, 1973; Prieto *et al.*, 2001), and can be induced by ionizing radiation and the action of certain gametocidal genes (Sears, 1956, 1993; Endo, 1988, 1990; Endo and Gill, 1996), the particular advantage of exploiting the *ph1b* mutant to promote allosyndesis is that the translocations are non-random: rather, they tend to involve the exchange of genetically related material. Its disadvantage is that the frequency of allosyndesis (and hence of recombination) is rather low, especially between chromosomes of more distantly related genomes such as *Triticum* and *Hordeum*. The level of *ph1b*-induced pairing between wheat and cereal rye (*Secale cereale*) chromosomes has been estimated to be around 4% (Miller *et al.*, 1994), which is about double the level noted here between the chromosomes of wheat and either of the two *Hordeum* spp. At the level of

recombination, however, an extensive *ph1b*-based attempt to reduce the length of the rye chromosome segment present in the widely used wheat/rye Robertsonian translocation 1BL.1RS resulted in an estimated recombination frequency of only around 0.7% (Koebner and Shepherd, 1986; Lukaszewski, 2000). The levels achievable in more closely related species, notably in the genus *Aegilops* (Koebner and Shepherd, 1987; Riley *et al.*, 1968a; Gill and Raupp, 1987; Farooq *et al.*, 1990; Ceoloni *et al.*, 1992), are much higher than this.

Our results showed that homoeologous recombination between *Hordeum* sp. and wheat chromosomes did only depend on the absence of the *Ph1* locus as no differences in the frequency of pairing were found when chromosome association in different homoeologous groups was studied. Most of chromosome associations between *Hordeum* sp. and wheat chromosomes were end-to-end extremely distal associations as described previously (Werner *et al.*, 1992; Benavente *et al.*, 1996; Calderón *et al.*, 2014).

In summary, the use of the *ph1b* mutant does induce a low, but significant level of chromosome pairing and recombination between wheat and *Hordeum* sp. chromosomes. The translocation and introgression chromosomes detected in the present work will serve as potential donor material for the breeding of cultivars having a higher grain carotenoid content, stronger resistance against *Septoria tritici* and improved salinity tolerance.

Chapter IV

Use of the wheat ph1b *mutant to introgress* Hordeum chilense *chromosome fragments to enhance carotenoid content in bread wheat*

In process:

Rey MD, Calderón MC, Rodrigo MJ, Zacarías L, Alós E, Prieto P. Use of wheat *ph1b* mutant to introgress *Hordeum chilense* chromosome fragments to enhance carotenoid content in bread wheat.

Abstract

The use of crop wild relative species to improve major crops performance is well established. *Hordeum chilense* has a high potential as a genetic donor to increase the carotenoid content of wheat. Genetic crosses between the 7**H**^{ch} *H. chilense* substitution lines in wheat and the wheat *ph1b* mutant allowed the development of wheat-*H. chilense* translocation lines for both 7**H**^{ch}α and 7**H**^{ch}β chromosome arms in the wheat background. These translocation lines were characterized by *in situ* hybridization and using molecular markers. In addition, HPLC analysis was carried out to estimate the carotenoid content and both 7**H**^{ch}α·7**A**L and 7**A**S·7**H**^{ch}β disomic translocation lines presented higher carotenoid content than the wheat-7**H**^{ch} addition line and double amount of carotenoids than the wheat itself. A proteomic analysis confirmed that the presence of chromosome 7**H**^{ch} introgressions in wheat did not alter the proteomic profile of the wheat flour. The *Psy1* gene, which is involved in the carotenoid biosynthetic pathway, was also cytogenetically mapped on the 7**H**^{ch}α chromosome arm. These new wheat-*H. chilense* translocation lines can be used as a powerful tool in wheat breeding programs to enrich the diet in antioxidants.

Keywords: *H. chilense*, carotenoid content, HPLC, Tyr-FISH, *Psy1* gene

Introduction

Wild species of bread wheat are important resources for broadening the genetic variability of crop plants and useful traits have been transferred from these species to wheat (Jiang *et al.,* 1994). *Hordeum chilense* is an extremely polymorphic diploid wild barley from South of America. It has high crossability with other members of the *Triticeae* tribe and presents several agronomical characteristics which could be transferred into wheat, such as high carotenoid content among others (Alvarez *et al.,* 1998; Forster *et al.,* 1990; Martín *et al.,* 1998; Rubiales *et al.,* 2000; Martín *et al.,* 2005). *Hordeum chilense* addition and substitution lines in wheat (Miller *et al.,* 1982; Islam *et al.,* 1978, 1981) are generally used as a bridge to generate wheat-*H. chilense* translocation or recombinant lines (Sears, 1972, 1981). However, pairing between wheat and related chromosomes from these species is rare (Islam and Shepherd, 1988). Chromosome pairing between homoeologous (related) chromosomes can be achieved using the *ph1b* mutant (Sears, 1981). The *Ph1* locus, which is located on the 5**B**L chromosome arm, ensures chromosome pairing and recombination between homologous (identical) chromosomes (Okamoto, 1957; Riley and Chapman, 1958; Sears and Okamoto, 1958; Sears, 1977). As it has been previously shown in this work (Chapter III), in the absence of the *Ph1* locus (*ph1b* mutant) unspecific chromosome associations can occur between related chromosomes. Therefore it is possible to transfer desirable agronomics traits from relatives species into wheat to improve wheat quality (Rey *et al.,* 2015, unpublished data) for example the carotenoid content, which is lower in bread wheat than other plant species (Halvorsen *et al.,* 2002).

Carotenoids are a diverse family of natural yellow and red pigments produced by autotrophic organisms (Farré *et al.,* 2010), extremely important in human and animal nutrition. Yellow pigments are often identified with two classes of carotenoids: carotenes, which are tetraterpenoid hydrocarbons, and

xanthophylls, which are carotenoids with one or more oxygenated groups in the molecule. Lutein is the main carotenoid found in wheat, and is, in most cases, accompanied by lower amounts of zeaxanthin, β-cryptoxanthin and β-carotene (Hentschel *et al.,* 2002; Kaneko *et al.,* 1995; Lepage and Sims, 1968). The chromosomal location of genes involved in carotenoid synthesis in *H. chilense* was deciphered using *H. chilense* addition lines in wheat (Alvarez *et al.,* 1998). The presence of chromosome 7**H**^{ch} of *H. chilense* increased the carotenoid content in wheat, and moreover, the ditelosomic addition line for 7**H**^{ch}α chromosome arm showed greater influence on the pigment content (Alvarez *et al.,* 1998). A chromosomal region on the distal part of chromosome 7**H**^{ch}α of *H. chilense* related to the carotenoid content has been recently reported (Rodríguez-Suarez *et al.,* 2012*b*). New genes controlling the carotenoid content were also found in the genome of *H. chilense*, such as *Carot1* and *Zds* (codifying for a zeta-carotene desaturase) genes, located on the centromeric region of chromosome 2**H**^{ch} and the *Psy1* (*Phytoene Synthase1*) gene, which was located on the 7**H**^{ch}*α* chromosome arm, (Atienza *et al.,* 2004, 2007; Rodríguez-Suárez *et al.,* 2012*b*). In fact, the enzyme PSY catalyses the first step of the carotenoid biosynthetic pathway and it is considered a limiting factor for carotenoid production (Li *et al.,* 2008).

Genomic *in situ* hybridization (GISH) is the most efficient and accurate technique to estimate the amount of alien chromatin introgressed in wheat (Schwarzacher *et al.,* 1989). Moreover, fluorescence *in situ* hybridization (FISH) combined with GISH enables the determination of the exact chromosomal compositions and resolutions of the chromosome arms involved in wheat-*H. chilense* translocations (Prieto *et al.,* 2001). *In situ* hybridization can be also used to physically map single-copy genes on mitotic chromosomes (van Gijlswijk *et al.,* 1997).

Classical genetic breeding can result in undesirable side-effects as a consequence of the alteration of the genomic composition. Thus it also important to evaluate the quality of the introgression lines produced by conventional breeding. Proteins constitute 10-15% of wheat grain dry weight and are mainly composed of storage proteins which include the gluten proteins, the major responsible of dough properties, and other minority proteins which might modify flour quality and/or be involved in hypersensivity reactions such as food allergy and celiac disease (De Angelis *et al.,* 2008; Nadolska-Orczyk *et al.,* 2009; Altenbach *et al.,* 2011; Larré *et al.,* 2011; Tasleem-Tahir *et al.,* 2011; Yadav and Singh, 2011). Hence, deciphering the composition of the endosperm proteins through proteomics approaches is useful to evaluate the potential interest of wheat introgression lines.

In this chapter, we describe the development of new wheat-*H. chilense* translocation lines for both 7$\mathbf{H}^{ch}\alpha$ and 7$\mathbf{H}^{ch}\beta$ chromosome arms in the wheat background, which have a substantial increment of the carotenoid content. In addition, the physical mapping of *Psy1 gene,* the first committed step in the carotenoid biosynthetic pathway, was carried out on the *H. chilense* chromosome 7$\mathbf{H}^{ch}$. An analysis of the proteomics profile of the flour of these new wheat-*H. chilense* translocation lines with a higher carotene content was also done.

Materials and methods

Plant material

Hordeum chilense substitution lines for chromosome 7$\mathbf{H}^{ch}$ in bread wheat (Miller *et al*, 1982) were used as parental lines in initial genetic crosses with the wheat line deficient for the *Ph1* locus (*Triticum aestivum* cv. Chinese Spring, *ph1bph1b* genotype; Sears, 1977). The descendence was backcrossed by the wheat *ph1b* mutant to obtain chromosome 7$\mathbf{H}^{ch}$ in the *ph1b* mutant background

as described in Figure 1. The seeds of *H. chilense* substitution lines for chromosome 7**H**^{ch} and of wheat line deficient for the *Ph1* locus were kindly supplied by Dr. Steve Reader (JIC, Norwich, UK). Seeds were germinated in Petri dishes on wet filter papers in darkness for 5 days at 4ºC followed by 24 hours incubation at 25ºC. Roots about 1 cm long were cut, incubated for 4 hours in a 0.05% colchicine solution at 25ºC and then fixed in 100% ethanol- acetic acid, 3:1 (v/v). Fixed roots were stored at 4ºC for at least 1 month to perform cytogenetic experiments. All plants were grown in a greenhouse at 26ºC (day) and 22ºC at night with a photoperiod of long days (16 h of daylight - 8 h of darkness).

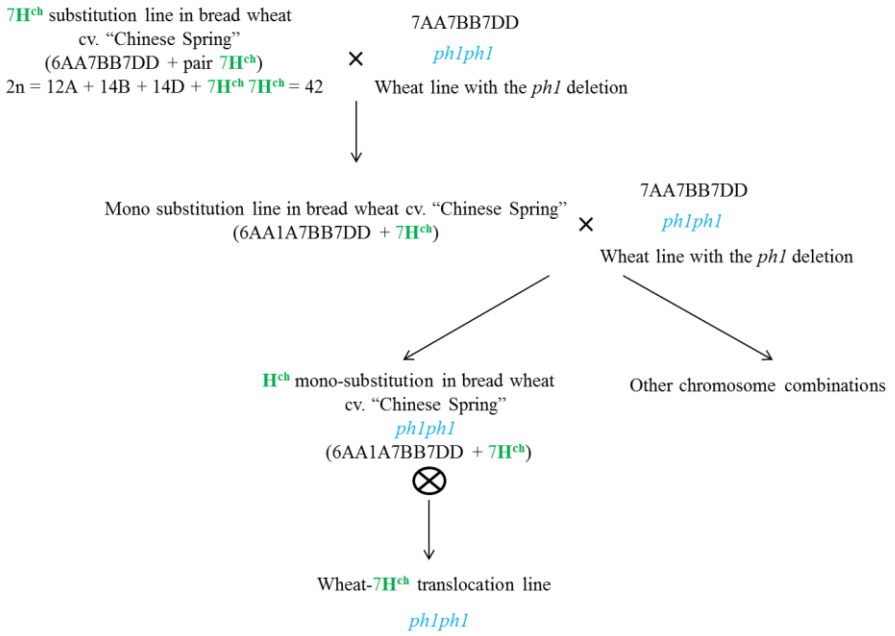

Figure 1. Development of *H. chilense* introgression lines in hexaploid wheat in the *ph1b* mutant background. Crosses between 7**H**^{ch} substitution line in bread wheat cv. Chinese Spring and the *ph1b* mutant in hexaploid wheat were developed and backcrossed to the *ph1b* mutant to obtain *Hordeum* translocation in the absence of the *Ph1* locus

Characterization of the translocation lines using molecular markers

Genomic DNA was extracted from young frozen leaf tissue using the CTAB method (Murray and Thomson, 1980) with some modifications according to (Hernández *et al.,* 2001). Bread wheat *ph1b* mutants were checked for the *ph1b* deletion using the ABC_{920} SCAR marker as previously described (Wang *et al.*, 2002). The PCR reaction were performed in 30 µl of reaction mixture containing 1x PCR buffer with $MgCl_2$ (Bioline USA, Taunton, MA), 0.25 mM dNTPs, 5 pmol primers, 0.02 U/ µl of Taq DNA polymerase (Bioline USA, Taunton, MA). PCR cocktail was initially denatured at 94ºC for 5 min, and then the amplification reaction consisted in 35 cycles of 1 min at 94ºC, 1 min at 51ºC and 1 min at 72ºC, followed by a final extension reaction of 7 min at 72ºC. PCR products were solved on 1% agarose gels in 1xTBE and visualized by ethidium bromide staining under UV light. The presence of both 7$\mathbf{H}^{ch}\alpha$ and 7$\mathbf{H}^{ch}\beta$ chromosome arms was analysed using the microsatellites BAWU763 and BAWU550, respectively, as described in Hagras *et al.,* (2005).

Cytogenetic analysis

GISH experiments were performed according to Prieto *et al,* (2004) using genomic *H. chilense* DNA as probe to confirm the presence of chromosome 7$\mathbf{H}^{ch}$. The identification of the 7$\mathbf{H}^{ch}\alpha$ or 7$\mathbf{H}^{ch}\beta$ chromosomes arms was also confirmed by FISH using the pAs1 sequences (Cabrera *et al.,* 1995; Rayburn and Gill, 1986). The wheat chromosome arms involved in inter-specific translocations with the *H. chilense* chromosome 7$\mathbf{H}^{ch}$ were also identified using both the GAA-satellite sequence (Pedersen *et al.,* 1996; Pedersen and Langridge 1997) and pAs1 probe (Rayburn and Gill, 1986) as described in Calderón *et al.,* (2012).

Physical mapping of *Psy1*

Physical localization of *Psy1* gene from the carotenoid biosynthetic pathway was performed by *in situ* hybridization. *Psy1* probe was prepared by PCR amplification of a 2538bp *Psy1* genomic region from hexaploid wheat. Primer pairs were designed using the Primer3plus software (Untergasser *et al.,* 2007) based on the *Psy1* sequence previously described in *H. chilense* (Atienza *et al.,* 2007; Rodríguez-Suárez *et al.,* 2012*a*; Zhang *et al.,* 2011). In detail, the sequences for the forward and reverse primers used for *Psy1* amplification were, 5'AGTGGTGAATCCATCCCTTG3' and 5'CCTTCCTCTTCTTGCACTGG3', respectively. PCR amplification for *Psy1* gene was performed using MyFi DNA polymerase (Bioline USA, Taunton, MA) according to the manufacturer´s instructions as follows: 3 min 94ºC, 35 cycles of 15 s at 94ºC, 15 s at 60ºC and 3.5 min at 72ºC. PCR products were resolved on 1% agarose gels in 1xTBE and stained with ethidium bromide and visualized under UV light. Chromosome spreads from root tips of germinated wheat seeds, probe labeling and *in situ* hybridization were carried as described by Prieto *et al.,* (2001). Detection of hybridization signals was carried out using the Tyramide Signal Amplification Kit (TSA™, PerkinElmer Life and Analytical Sciences, Inc., Waltham, MA, USA). To identify wheat chromosomes with positive signals, samples were re-hybridized using the pAs1 repetitive sequence and GAA-satellite sequence as probes (Rayburn and Gill, 1986; Pedersen *et al.,* 1996). Individual slides were observed under a Nikon Eclipse 80i, microscope (Nikon Instruments Europe BV, UK). Images were captured with a Nikon CCD camera using the appropriate Nikon 3.0 software and processed with Photoshop 4.0 software (Adobe Systems Inc., San Jose, California, USA).

Analysis of the carotenoid content in wheat-*H. chilense* translocation lines

Carotenoids from developing grains were determined according to Rodríguez-Suárez *et al.*, (2014). For each sample, 4 grams of each line were reduced to fine flour in a mortar. Between 0.5-1 gr. of flour per replica was extracted to analysis the content of carotenoids in grains. The identification and quantification of individual carotenoids was carried out using a high performance liquid chromatography (HPLC) with diode array detector described by Carmona *et al.*, (2012). Three replicates were analysed per sample.

Statistical analysis

Statistical analyses were performed using STATISTIX 9.0 software (Analytical Software, Tallahassee, FL, USA). The analysis of variance (ANOVA) was based on randomized blocks. Means were separated using the Least Significant Difference (LSD) test with a probability level of 0.05.

Protein extraction and quantification

Proteins were extracted following a phenol-based protocol described in Collado-Romero *et al.*, (2014) with slight modifications. Briefly, from each genotype two independent samples composed of a pool of 2-3 seeds was ground into a fine powder using a ball mill (Star-Beater, VWR). The ground tissue was resuspended in phenol extraction buffer (0.9 M sucrose, 0.5 M Tris-HCl, 50 mM EDTA, 0.1 M KCl, Milli-Q water and freshly added 1% Triton X-100, 2% β-mercaptoethanol and 1% protease inhibitor cocktail set VI (Calbiochem), pH 8) and homogenized on ice using Eppendorf micropestles. Samples were subsequently mixed with one volume of phenol solution equilibrated with 10 mM Tris HCl pH 8, 1 mM EDTA (Sigma-Aldrich), shaken for 1 min, incubated for 20 min in a tube rotator at 4 ºC and centrifuged at 18000 × *g* for 10 min at 4

ºC. The upper phenolic phase was collected and proteins were precipitated by adding five volumes of ice cold 0.1 M ammonium acetate and 13 mM DTT in methanol at -80 ºC for 2 h. A pellet of proteins was obtained by centrifugation at 20000 × *g* for 20 min at 4 ºC. Then, the pellet was washed once with ice cold 0.1 M ammonium acetate, 13 mM DTT in methanol and twice with 80% ice cold acetone. Finally, the pellet was air dried, dissolved in denaturing buffer (6 M urea, 50 mM ammonium bicarbonate pH 8) and stored at -80 ºC. Protein concentration was determined with the Pierce BCA Protein Assay Kit (Cultek), using BSA as a standard according to manufacturer's instructions for the microplate procedure. Protein quality was checked by 1D-SDS-PAGE using Mini-Protean cell (Bio-Rad Laboratories, Spain) and 12% Mini-PROTEAN® TGX™ precast polyacrylamide gels (Bio-Rad) stained with Coomassie Blue G250.

Reverse phase-liquid chromatography RP-LC-MS/MS analysis

Protein extracts in 6 M urea and 50 mM ammonium bicarbonate pH 8 were reduced and alkylated. Disulfide bonds from cysteinyl residues were reduced with 10 mM DTT for 1 h at 37 ºC, and then thiol groups were alkylated with 50 mM iodoacetamide for 1 h at room temperature in the dark. Samples were diluted to reduce urea concentrations below 1.4 M and digested using sequencing grade trypsin (Promega, Madison, WI) overnight at 37 ºC in a trypsin/protein ratio of 1:5 (w/w). Digestion was stopped by the addition of 1% TFA. Then, the supernatants were dried down and desalted onto ZipTip C18 Pipette tips (EMD Millipore Corporation, Billerica, MA) until mass spectrometric analysis.

Desalted digested proteins were dried out, resuspended in 0.1% formic acid and analyzed by RP-LC-MS/MS in an Easy-nLC II system coupled to an ion trap LTQ-Orbitrap-Velos-Pro mass spectrometer (Thermo Fisher Scientific Inc.,

Waltham, MA). The peptides were concentrated (on-line) by reverse phase chromatography using a 0.1 mm × 20 mm C18 RP precolumn (Acclaim PepMap100 nanoViper, Dionex), and then separated using a 0.075 mm × 100 mm C18 RP column (Acclaim PepMap100 nanoViper, Dionex operating at 0.3 µl/min.

Peptides from a 5 µg aliquot of the protein extract were eluted in a 180-min gradient of 5 to 40% solvent B (solvent A: 0.1% formic acid in water, solvent B: 0.1% formic acid, 80% acetonitrile in water). ESI ionization was carried out using a Nano-bore emitters Stainless Steel ID 30 µm (Proxeon) interface. The Orbitrap resolution was set at 30.000. Peptides were detected in survey scans from 400 to 1600 amu (1 µscan), followed by twenty data dependent MS/MS scans (Top 20), using an isolation width of 2 u (in mass-to-charge ratio units), normalized collision energy of 35%, and dynamic exclusion mode applied during 30 s periods. Peptide identification from raw data was carried out using the SEQUEST algorithm (Proteome Discoverer 1.4, Thermo Scientific). Database search was performed against Uniprot_Viridiplantae. The following constraints were used for the searches: tryptic cleavage after Arg and Lys, up to two missed cleavage sites, and tolerances of 10 ppm for precursor ions and 0.8 Da for MS/MS fragment ions. Searches were performed allowing optional Met oxidation and Cys carbamidomethylation. Search against decoy database (integrated decoy approach) was performed using false discovery rate (FDR) < 0.01. Protein identification by nLC-MS/MS was carried out at the CBMSO protein chemistry facility, a member of ProteoRed network.

Bioinformatics and functional analysis of identified proteins

The output accessions obtained with the Proteome Discoverer software were exported to Microsoft Excel for data analysis. Firstly, a table containing information of all the proteins identified in the four genotypes analyzed was

generated (Table S2). The data obtained from the Uniprot-Viridiplantae search revealed that there were 372 proteins whose best hit was a protein with unknown function, meaning 50% of the proteins identified. Hence, to improve the information about the peptides matching proteins with unknown function a manual blastp was carried out. This analysis consisted on the blastp of the protein with unknown function with the Uniprot database; this allowed the identification of highly homologous proteins with an assigned function (identity with the protein with the best hit and the protein with described function > 80%).

In addition, a table containing the proteins exclusively identified in the genotypes with increased carotenoid content was created (Table 2). To this end, only the proteins that were present in the two replicates of each line were considered for the comparison between lines. Exceptionally, interesting proteins that were not exclusively found in one of the lines or in the two replicates of the proteomics experiments were also included in the list because they could have a relationship with the accumulation of carotenoids. These exceptions are indicated in the table and marked with asterisks.

Results

Development of wheat-chromosome 7H^{ch} translocation lines in hexaploid wheat

Genetic crosses between chromosome 7**H**^{ch} addition line in wheat and the *ph1b* mutant in hexaploid wheat were carried out with the aim to introgress chromosome 7**H**^{ch} into wheat, promote interspecific chromosome associations between chromosome 7**H**^{ch} and its wheat homoeologous in the *ph1b* mutant background and reduce the size of chromosome 7**H**^{ch} in the wheat background (Figure 1). Screening and characterization of plants carrying introgressions from *H. chilense* chromosome 7**H**^{ch} were carried out by molecular markers and

multicolor *in situ* hybridization. BAWU550 and BAWU763 microsatellites were used to identify plants carrying chromosome 7$\mathbf{H}^{\mathbf{ch}}$ introgressions (Figure 2). The presence of both molecular markers indicated the presence of the whole chromosome 7$\mathbf{H}^{\mathbf{ch}}$ but could not discern between a whole chromosome introgression or reciprocal chromosome translocations between the *H. chilense* and the wheat homoeologous chromosomes. Translocations between chromosome 7$\mathbf{H}^{\mathbf{ch}}$ and wheat chromosomes were detected by GISH (Figure 3). Combined GISH and FISH experiments enabled the determination of the exact chromosomal compositions and resolution of the exact chromosome arms involved in wheat-chromosome 7$\mathbf{H}^{\mathbf{ch}}$ translocations (Figure 3). Thus heterozygous 7$\mathbf{H}^{\mathbf{ch}}$α·7**A**L and 7**A**S·7$\mathbf{H}^{\mathbf{ch}}$β robertsonian translocations were only detected by *in situ* hybridization. Homozygous 7$\mathbf{H}^{\mathbf{ch}}$α·7**A**L and 7**A**S·7$\mathbf{H}^{\mathbf{ch}}$β translocations in the wheat background were obtained in the final selfed population.

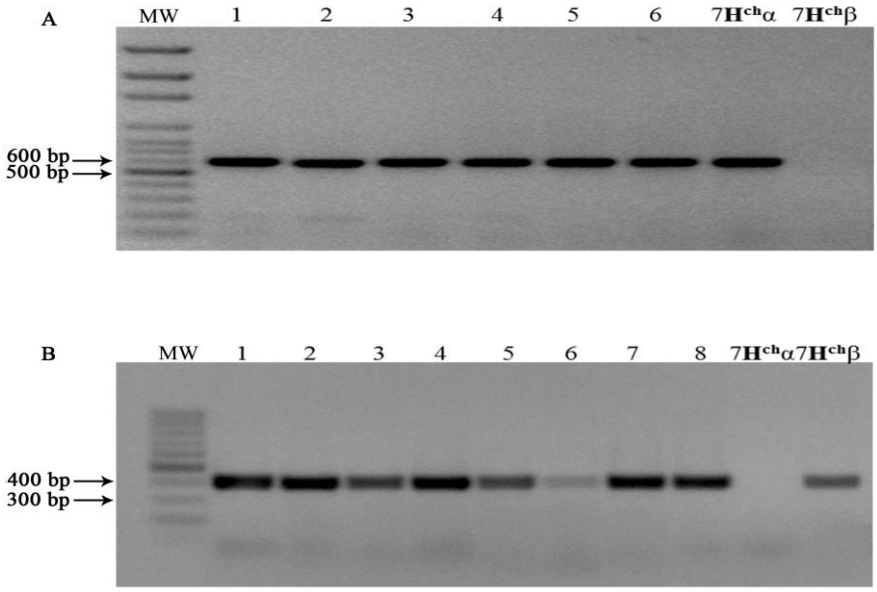

Figure 2. The PCR amplification for genomic DNA of translocations lines between chromosome 7H^{ch} and wheat chromosomes. A. The presence of small arm of chromosome 7**H**^{ch} is amplified for BAWU763 EST fragment. B. The presence of long arm of chromosome 7**H**^{ch} is amplified for BAWU550 EST fragment.

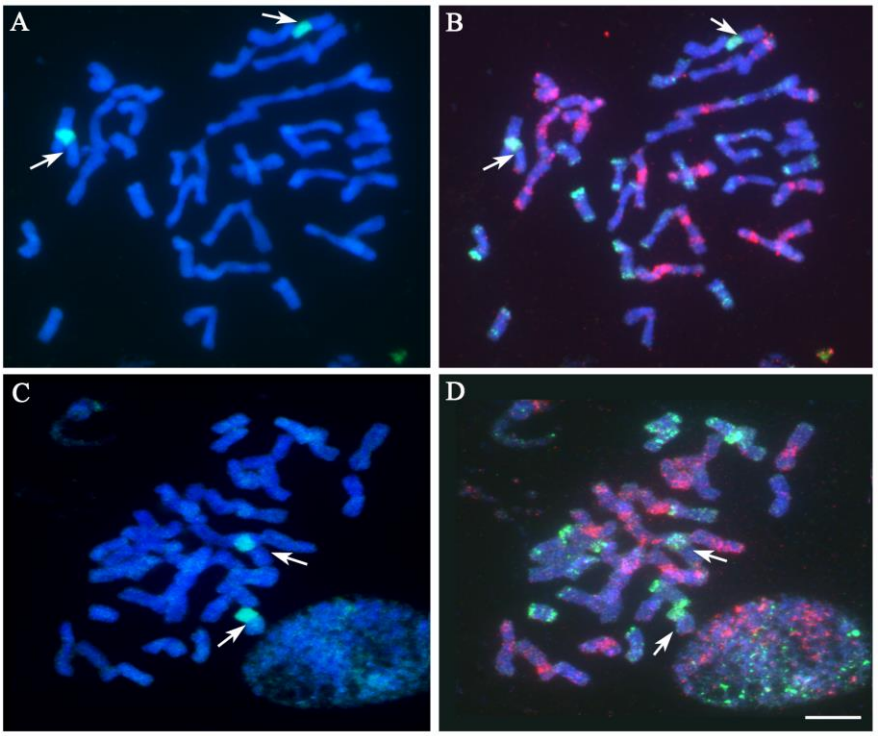

Figure 3. Translocation lines involving chromosome 7H^{ch} from *H. chilense* in the bread wheat background. A. GISH and B. FISH pattern of a mitotic metaphase carrying two copies of 7**H**^{ch}α-7**A**L Robertsonian translocation. C. GISH and D. FISH pattern of a mitotic metaphase carrying two copies of 7**A**S-7**H**^{ch}β Robertsonian translocation. Bar: 10μm.

Physical mapping of *Psy1* gene in *H. chilense* chromosome 7H^{ch} introgression lines in hexaploid wheat

Based on the *Psy1* DNA sequence, primers were designed as described in the materials and methods section, to amplify a 2538bp fragment of the *Psy1* gene in *H. chilense* (Figure 4). The physical localization of *Psy1* gene was performed by fluorescence *in situ* hybridization using the 2538bp *Pys1* genomic DNA sequence as a probe. *Psy1* locus was visualized on *H. chilense* 7**H**^{ch}α chromosome arm (Figure 4). No signals were detected on the homoeologous wheat chromosomes.

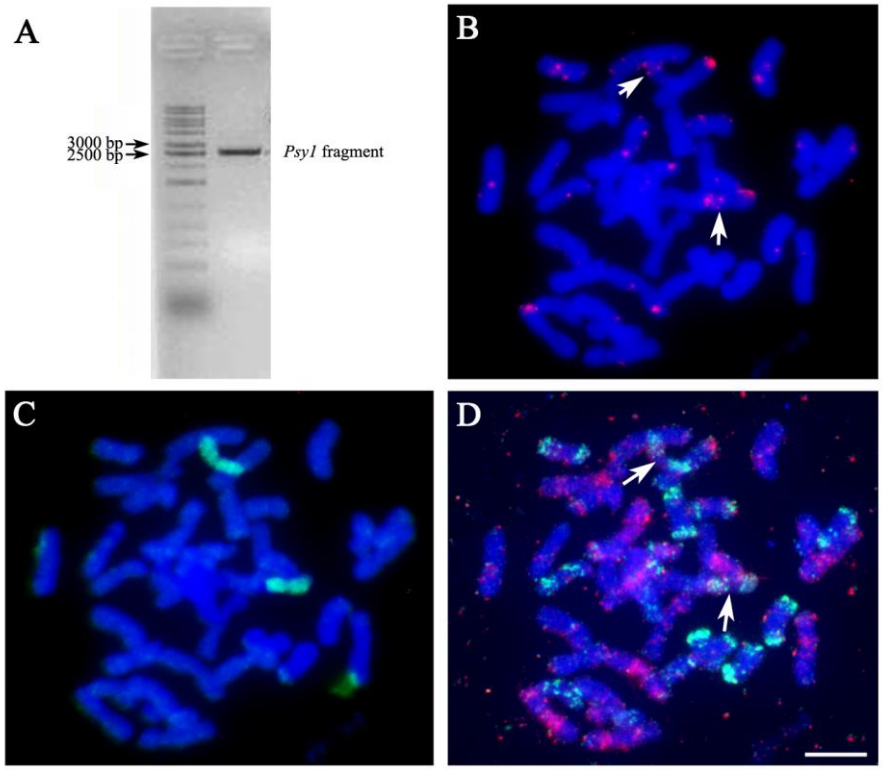

Figure 4. Tyr-FISH results showing the sequence (2538bp) of *Psy1* gene in 7H^{ch} substitution line in *Triticum aestivum* cv. Chinese Spring. A. The DNA was counterstained with DAPI (blue). B. A metaphase I of 7**H**^{ch} (7**A**) disomic wheat-barley substitution line showing Tyr-FISH signals localized on metacentric chromosomes of 7**H**^{ch} (arrowed). C. Genomic *in situ* hybridization (GISH) on the same metaphase chromosomes of 7**H**^{ch} (7**A**) disomic wheat-barley substitution line. D. Multicolor fluorescence *in situ* hybridization (FISH) on the metaphase I chromosomes of 7**H**^{ch} (7**A**) disomic wheat-barley substitution line. Bar: 10μm.

Analysis of the carotenoid content in wheat-*H. chilense* translocation lines

The carotenoid content was determined in *H. chilense* translocation lines for chromosome 7**H**^{ch} in wheat and compared to wheat. The main carotenoids identified in all samples were lutein (free and esterified) and zeaxanthin. The quantification of individual carotenoids and the amount of total carotenoids are showed in Table 1. The total carotenoids (1133.5 ± 68.5) content in 7**H**^{ch}α·7**A**L

translocation line was almost double than the wheat control and similar to the one in 7**A**S·7**H**^{ch}β translocation line (1215 ± 13.5). As expected, the carotenoid content of the bread flour was the lowest (603 ± 59), followed by the chromosome 7**H**^{ch} addition line in bread wheat (803 ± 58). Thus, the maximum carotenoid content was detected in any of the translocation lines for both 7**H**^{ch}α or 7**H**^{ch}β chromosome arms in the background of *ph1b* mutant. The maximum content for both free lutein and trans-zeathantine was detected in T7**H**^{ch}α·7**A**L translocation line, although this line showed the minimum content in esterified lutein. However, the long arm translocation line showed the lower of trans-zeathantine content. Therefore, our results clearly indicate that the new translocation lines generated showed higher carotenoid content than both, bread wheat and the wheat line carrying the addition of the whole chromosome 7**H**^{ch}.

Lines	Total carotenoids (ng/g FW)	Free lutein		Esterified lutein		Trans-zeathantin	
		ng/g FW	%	ng/g FW	%	ng/g FW	%
Bread wheat	603 ± 59*c*	321 ± 39*b*	53.23	70 ± 13*c*	11.60	213 ± 6*ab*	35.32
Wheat-7**H**^{ch} disomic addition	803 ± 58*b*	326 ± 17*b*	40.60	268 ± 25*a*	33.37	209 ± 15*b*	26.02
7**H**^{ch}α·7**A**L disomic translocation	1133 ± 68*a*	874 ± 43*a*	77.10	23 ± 5*d*	2.10	235 ± 30*a*	20.80
7**A**S·7**H**^{ch}β disomic translocation	1215 ± 13*a*	844 ± 17*a*	69.47	176 ± 12*b*	14.47	195 ± 14*c*	16.08

Table 1. Carotenoid content (ng per grams of fresh weight, ng/g FW) in bread wheat, wheat-7H^{ch} disomic addition, 7H^{ch}α·7AL and 7AS·7H^{ch}β disomic translocation lines. Data are mean ± SE of three biological replicates. The letters in italics indicate statistical significance ($P < 0.05$).

Comparison of the seed proteomic profile among wheat and the introgression lines with carotenoid-enriched seeds

Seed proteins were extracted from *Triticum aestivum* cv. Chinese Spring and three introgression lines with carotenoid enriched-seeds, namely: a wheat line with the addition of 7**H**^{ch}, a line with the translocation of the α arm of 7**H**^{ch} and another line with the translocation of the β arm of 7**H**^{ch}. The protein extraction protocol consisted on the extraction from two replicates of each line with a phenol-based buffer followed by precipitation with ammonium acetate. The quality and the complexity of the extracted proteins were checked by 1D-SDS-PAGE prior to nLC-MS/MS. The band pattern of the seed extracts was highly similar among lines (Figure 5). Then, a high sensitive system of reverse-phase nLC coupled to a high resolution and mass accuracy mass spectrometer (LTQ-Orbitrap-Velos-Pro) was used to analyse the samples. To minimize the number of false positives or misidentifications a high level of confidence was applied for protein identification. Thus, only peptides with 5 to 30 amino acids and a minimum of two peptides per protein were required for positive identification. A false discovery rate (FDR) < 0.01 was also set. As a result, 741 different proteins were identified from all the seed extracts analysed (Table S1, **https://www.dropbox.com/s/kz00oz97s4giv91/Supplementary%20Table%20S1.xlsx?dl=0**).

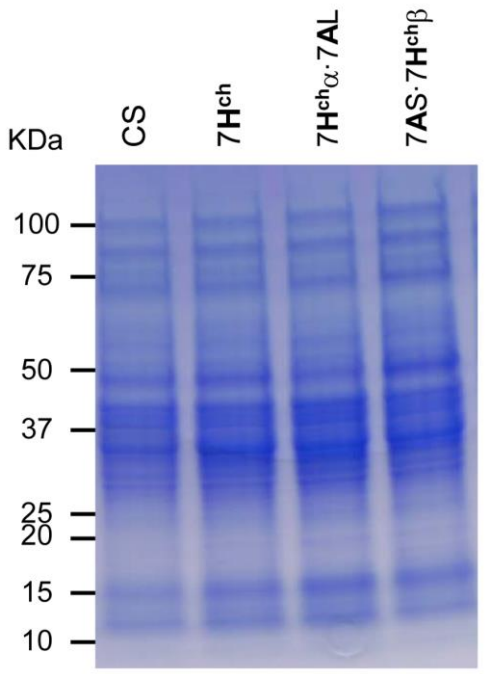

Figure 5. SDS-PAGE stained with Coomassie Brilliant Blue G250 of the seed protein extracts obtained from bread wheat (CS, lane 1), wheat-7H^{ch} disomic addition line (lane 2), and the 7H^{ch}α·7AL (lane 3) and 7AS·7H^{ch}β (lane 4) disomic translocation lines.

For a more astringent and reliable evaluation of the results, only the proteins that were present in the two replicates of each line were considered for further analysis. Hence, 368 proteins were consistently identified in the two replicates of all the extracts analysed. Ninety percent of the proteins (328) were common to the CS and the introgression lines, and only ten percent of the proteins (40) were specifically present in some of the lines (Figure 6).

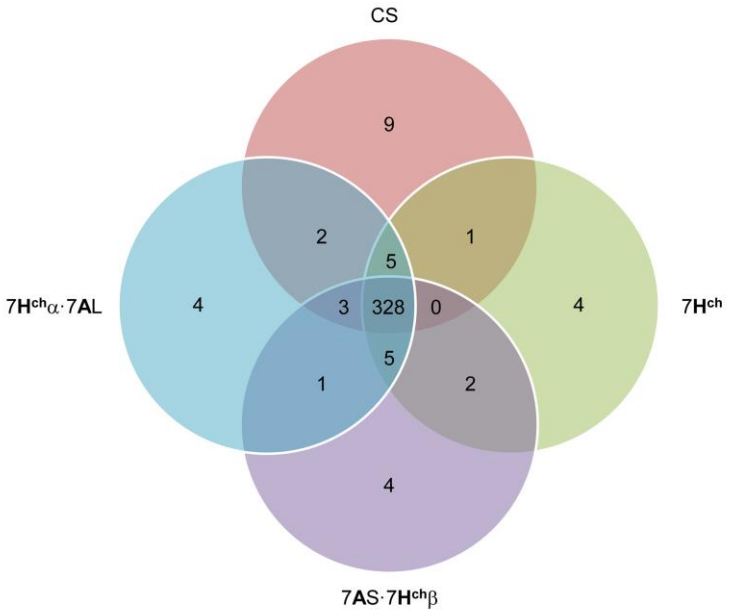

Figure 6. Venn diagram summarizing the proteins identified in seed extracts of bread wheat, wheat-7H^{ch} disomic addition line, and the 7H^{ch}α·7AL and 7AS·7H^{ch}β disomic translocation lines. Only peptides with 5 to 30 amino acids and a minimum of two peptides per protein were required for positive identification, and peptide FDR < 0.01.

There were 12 proteins which were only identified in either the addition or in each of the translocation lines but not in the wild type. Out of them 7 proteins had as best hit a protein with unknown function, therefore to increase the information about these proteins manual blastp were performed in an attempt to find highly similar proteins with an assigned function. Only those showing higher identity than 80% were considered (Table S1).

The search for proteins with functions that could be possibly related to the regulation of carotenoid accumulation was carried out by searching at the whole set of proteins that were not present in bread wheat but in some of the other lines with higher carotenoid contents. This analysis led to the selection of a 14-3-3 protein, a small heat shock protein (sHSP, 26.4 kDa), a Heat-shock protein

70KDa and a HSP70-HSP90 organizing protein (O49996, A5A8UA, M5WX60 and D2E9R6, respectively). The 26.4 kDa HSP was present in one replicate of the translocation of the 7$\mathbf{H}^{ch}\alpha$ chromosome and in the two replicates of addition 7$\mathbf{H}^{ch}$. Heat-shock protein 70KDa was found in one replicate of the translocation of the 7$\mathbf{H}^{ch}\alpha$ and 7$\mathbf{H}^{ch}\beta$ and HSP70-HSP90 organizing protein was found in the two replicates of the of the translocation of the 7$\mathbf{H}^{ch}\alpha$ (Table 2).

Line	Uniprot ID	Protein name
Addition 7H^{ch}	O49996	**14-3-3-like protein**
	F2CX17	Predicted protein (88% identity with cold shock domain protein 2; Q75QN9)
	W5FAI9	Uncharacterized protein (100% identity with defensin; A0A060AQ78)
	R7W8W0	Defensin-like protein 1
	A5A8U9	**26.4kDa heat-shock protein***
7H^{ch}α·7AL	**D2E9R6**	**Hsp organizing protein/stress-inducible protein**
	M7YCT7	3-ketoacyl-CoA thiolase 2, peroxisomal
	A2YP75	Putative uncharacterized protein
	W5A1H5	Uncharacterized protein
	A5A8U9	**26.4kDa heat-shock protein***
7AS·7H^{ch}β	Q2QLR2	Glycine-rich RNA-binding protein GRP1A
	I1QDX3	Uncharacterized protein (99 % identity with 2,3-bisphosphoglycerate-independent phosphoglycerate mutase; Q10LY9)
	F2E2F1	Predicted protein (100 % identity with 60S ribosomal protein L21-2; M8CY06)
	F2CSZ7	Predicted protein
	M5WX60	Uncharacterized protein (85% identity with Heat shock 70 kDa protein 15; W9R6E1)**
7H^{ch}α·7AL and 7AS·7H^{ch}β	Q39782	Alcohol dehydrogenase 2a
Addition 7H^{ch} and 7AS·7H^{ch}α	F2D712	Predicted protein
	W5GCI3	Uncharacterized protein
Addition 7H^{ch}	B9VUV5	Low molecular weight glutenin subunit

and 7**H**^{ch}α·**7A**L and 7**A**S·7**H**^{ch}β	Q1ZZT4	Low molecular-weight glutenin subunit
	Q6J162	S-type low molecular weight glutenin
	K4AAT0	Uncharacterized protein (83% identify with Serpin-ZXA; Q75H81)
	M8BX24	Uncharacterized protein

Table 2. List of proteins exclusively identified in the protein extracts of the lines with enhanced carotenoid accumulation. Unless otherwise stated the proteins were identified in the two replicates of the lines and not in any other protein extracts. The Uniprot identification number (ID; http://www.uniprot.org/) and the protein name of the best matches of the identified peptides are included. When the best match corresponded to a protein with a yet unassigned function the protein with the highest homology (>80% identity) was also indicated in brackets. The proteins with a possible implication in carotenoid enrichment are highlighted in bold.

Discussion

Most mapping studies in wheat agree that QTLs located on group 7 chromosomes largely determine the yellow pigment content of the grains (YPC). And *Psy1*, the first committed step in the carotenoid biosynthetic pathway, was considered a candidate gene to explain the YPC of wheat grain since it maps to chromosomes 7**A** and 7**B** of durum and bread wheat (reviewed in Rodríguez-Suárez *et al.,* 2010).

Tritordeums, which are amphiploids obtained after chromosome doubling of wheat and *Hordeum chilense*, have higher carotenoid pigment contents than durum or bread wheat (Atienza *et al.,* 2007). The analysis of pigment content of the flour from wheat-*H. chilense* addition lines with single chromosomes led to the conclusion that chromosome 7$\mathbf{H^{ch}}$ from *H. chilense* confers the capacity to accumulate higher carotene concentration in seeds (Alvarez *et al.,* 1998). Moreover, the *Psy1* gene is the only gene related with the carotenoid biosynthetic pathway physically mapped in *H. chilense* (Li *et al.,* 2008). Taking into account all this information, we developed genetic crosses between the (7**A**)7$\mathbf{H^{ch}}$ substitution line in wheat and the wheat *ph1b* mutant to facilitate chromosome associations and recombination between chromosome 7$\mathbf{H^{ch}}$ and those from the wheat homoeologous group 7. Homozygous 7$\mathbf{H^{ch}}$α·7**A**L and 7**A**S·7$\mathbf{H^{ch}}$β translocation lines in hexaploid wheat were obtained and the evaluation of the pigment content in this translocation lines was carried out. The 7$\mathbf{H^{ch}}$α·7**A**L translocation lines showed higher carotenoid content than bread wheat as expected because as *Phy1* gene is located in 7$\mathbf{H^{ch}}$α chromosome arm from *H. chilense* (Rodriguez-Suárez *et al.,* 2012*b*). We have cytogenetically mapped this *Psy1* locus on 7$\mathbf{H^{ch}}$α chromosome arm in a (7**A**)7$\mathbf{H^{ch}}$ substitution line in bread wheat using the biotinyl tyramide system (Figure 4). This gene seemed to be specific from *H. chilense* as no signals were detected in any of the wheat chromosomes 7**A**, 7**B** or 7**D** from the wheat related genomes. However,

the 7**A**S·7**H**^{ch}β translocation line also showed higher total carotenoid content than the wheat control and similar to the 7**H**^{ch}α·7**A**L. The higher carotenoid levels in the 7**A**S·7**H**^{ch}β line can be related to the presence of a QTL in the distal part of the 7**H**^{ch}β chromosome arm associated with the increment of YPC. However, so far there are no candidate genes described in this region related to YPC (Rodríguez-Suárez *et al.*, 2012*b*).

Comparison of PSY predicted protein sequences from selected Australian wheat genotypes suggested that differences in putative sites for post-translational modification may influence enzyme activity and subsequent xanthophyll accumulation in the wheat endosperm (Howitt *et al.,* 2009). Hence, proteins that modify the enzymes involved in carotenoid biosynthesis are interesting candidates as potential key regulators of carotenoid content in seeds.

The proteomics analysis comparing the endosperm proteome of the addition of 7**H**^{ch} and the translocation of the 7**H**^{ch}α and 7**H**^{ch}β chromosome arms pointed out the presence of 14-3-3 and heat shock protein (HSPs). Both 14-3-3 and HSP70 proteins were previously described to be required for the translocation of nucleus-encoded chloroplast precursor proteins into the chloroplast (May and Soll, 2000) which seemed to indicate that these proteins could somehow improve protein trafficking to the plastids of wheat endosperm and consequently enhance carotenoid accumulation or biosynthesis. For example, plant DXP reductoisomerase (DXR) which catalyses the second step in the MEP pathway has an N-terminal transit domain with a putative motif for a 14-3-3 binding site (Fung *et al.*, 2010). Therefore, the post-translational modifications of biosynthetic proteins due to the interaction with 14-3-3 proteins and/or HSP70 could be involved in the accumulation of carotenoids observed in the introgressed lines (Table 1). Furthermore, several studies in tomato fruits have revealed that HSPs are related to carotenoid accumulation. Hence, constitutive

expression of *HSP21* promoted carotenoid (lycopene) accumulation in developing tomato fruits (Neta-Sharir *et al.,* 2005) and interestingly, *HSP21* and *HSP70* were down-regulated in fruits of *AP2* RNAi lines with altered carotenoid accumulation (Karlova *et al.,* 2011). The 26.4 KDa heat-shock protein (A5A8U9), which was present in the lines with the addition of 7**H**^{ch} and the 7**A**S·7**H**^{ch}β translocation line, could also be playing a key role in the accumulation of carotenoids. In fact, 26.4 KDa heat-shock protein is highly similar to HSP21, also has a chloroplast peptide (http://www.cbs.dtu.dk/services/ChloroP) and contains an alpha-crystallin domain. Additionally, other authors have found out that small heat shock proteins were the most abundant proteins present in the carotenoid-protein complexes of cassava roots suggesting their involvement in the accumulation of these pigments. Moreover, an isoform of *HSP21* possessing four single point mutations in a variety with intense yellow cassava storage root may be responsible for increased sequestration of carotenoids (Carvalho *et al.,* 2012).

To summarize, the comparison of the proteomic profile of the wheat introgression lines with the wild-type revealed that the overall protein content was scarcely altered by the introgression of *H. chilense* chromosome 7**H**^{ch} or 7**H**^{ch}α and 7**H**^{ch}β chromosome arms and suggested that HSPs and a 14-3-3-like protein could play a key role in the enhancement of carotenoid accumulation in seeds (Figure 5, Table 2).

The translocation lines developed in this chapter are an important tool to enrich the carotenoid content in bread wheat. In addition, there is not available neither substitution/addition line nor translocation line for chromosome 7**H**^{ch} in durum wheat. Thus, these translocation lines are also a useful tool to transfer these chromosome arms into durum wheat, and therefore, enrich durum wheat in carotenoid content.

Chapter V

A rapid assay to detect alien genetic introgressions in bread wheat by dot-blot hybridization

In process:

Rey MD, Prieto P. A rapid assay to detect alien genetic introgressions in bread wheat by dot-blot hybridization.

Abstract

An efficient and user-friendly method for the screening of small random genomic introgressions from an alien species into wheat germplasm has been developed in the present work. The use of genomic dot blot hybridization allowed the detection of small *H. chilense* genomic introgressions from the descendence of genetic crosses between *H. chilense* addition or substitution lines in wheat and the wheat *ph1b* mutants. Based on the genomic *in situ* hybridization, slot blots from wheat lines carrying putatively *H. chilense* introgressions were immobilized on the membrane, blocked with wheat genomic DNA and hybridize using labeled *H. chilense* genomic DNA as a probe. This technique allowed a rapid and reliable screening for detecting small random *H. chilense* genomic introgressions introduced during a breeding program in the background of bread wheat when molecular markers cannot be used. Thus, the number of plants to be analyzed by molecular markers or *in situ* hybridization decreased, saving time and money. The technique was sensitive enough to detect a minimum of 5 ng of total genomic DNA or an about 1/420 dilution of *H. chilense* DNA when it is introgressed in the wheat background. The robustness of the technique was checked by *in situ* hybridization, revealing that all positive signals in the dot blot assay corresponded to wheat lines carrying any *H. chilense* genomic introgressions. The detection of other wheat relative species such as *Hordeum vulgare*, *Secale cereale* and *Agropyron cristatum* in the wheat background is also reported.

Keywords: Dot-blot hybridization, genomic introgressions, *H. chilense*, bread wheat

Introduction

The introgression of genetic material from wild or distantly related species into wheat germplasm is a classical and effective approach for broadening the genetic basis of this crop. Bread wheat-related species hybridization makes possible the transference of agronomically useful genes from those relative species into wheat. For example, wheat is influenced by several fungal diseases, biototrophic fungi cause the leaf and stripe rust diseases and powdery mildew or necrotrophic fungi such as *Septoria tritici* and *Fusarium graminearum* (Duveiller *et al.,* 2007). Fortunately, there are different relative species which can be used as genetic tools to transfer resistance genes for these diseases into wheat, such as *Hordeum* species. For example, there are genes conferring resistance to powdery mildew on *H. vulgare* chromosome 1$\mathbf{H^{v}}$ (Graner *et al*. 1991); genes conferring resistance to *Puccinia graminis* on *H. vulgare* chromosomes 5$\mathbf{H^{v}}$ and 7$\mathbf{H^{v}}$ (Kleinhofs *et al.,* 1993; Borovkova *et al.,* 1995) and genes conferring tolerance to greenbug (*Schizaphis graminum* Rond.) on chromosoma 7$\mathbf{H^{ch}}$ from *H. chilense* (Castro *et al.,* 2011). *Hordeum chilense* chromosome addition and substitution lines were developed in bread wheat and used for the transfer of wild barley genes into wheat (Miller *et al.,* 1982). Similar cytogenetic stocks have been developed involving the cultivated barley (*H. vulgare*) (Islam *et al.,* 1978, 1981) and the rye (*Secale cereale* L.) chromosomes (Chapman and Riley, 1955; Riley and Chapman, 1958a; Miller, 1984).

Wheat chromosomes and those of its relatives fail to pair with one another. This failure of homoeologous chromosomes to interact at meiosis is due to the *Ph1* locus (Okamoto, 1957; Riley and Chapman, 1958; Sears and Okamoto, 1958; Sears, 1977). Since the characterization of the *Ph1* locus, its absence (*ph1b* mutant) has been used widely and successfully in wheat to induce

homoeologous recombination (Sears, 1977, 1981, 1982; Riley *et al.*, 1968; Lukaszewski, 2000; Qi *et al.*, 2008; Liu *et al.*, 2011; Zhao *et al.*, 2013). In the absence of the *Ph1* locus, all chromosomes can remodel without the requirement for the presence of an identical or near identical chromosome, and this increases the chance of pairing between related and wheat chromosomes (Prieto *et al.*, 2004a; Rey *et al.*, 2015 unpublished data, Lukaszewski, 2000). In fact, the use of the *ph1b* mutant allowed reducing the linkage drag of the relative species in the wheat background and obtaining recombinants between those relatives and bread wheat (Lukaszewski, 2000; Rey *et al.*, 2015 unpublished data). Other methods have been used to introgress desirable characters from related species into bread wheat. The gametocidal genes (Gc genes) of *Aegilops cylindrical* Host. have been used to develop barley-wheat translocation lines (Endo *et al.*, 1998), and wheat-barley translocation lines have been derivates of hybrids multiplied *in vitro* (Mólnar-Láng *et al.*, 2000). However, these methods are random and the linkage drag is not reduced. Recombination can be restricted to homoeologous chromosomes using the *ph1b* mutant although crossovers still occur randomly between homoeologous chromosomes.

Identification of alien genomic introgressions can be difficult, especially when the genomic introgressions have occurred randomly. It is also limited by the complexity of the wheat genome and the high level of synteny among related species (Salse and Feuillet, 2007). The use of molecular markers combined with *in situ* hybridization is very useful to find exogenous genetic introgressions (Schwarzacher *et al.*, 1989, Calderón *et al*., 2012; Zhao *et al.*, 2013), but the exogenous chromosome fragment needs to be well characterized in order to choose specific molecular marker to be unequivocally distinguished from the equivalent chromosome region in related species. In addition, *in situ* hybridization enables the determination of the exact chromosomal compositions of the chromosome arms involved in wheat-*H. chilense* translocations (Prieto *et*

al., 2001). But cytogenetic experiments require high expertise and a long time what makes the cytogenetic approach expensive when there is a need of analyzing hundreds of plants. Thus breeders must be provided with methods of assessment rapid and that can be routinely applied when large numbers of plants have to be screened. Dot blot hybridization has been used for many years as a routine assay to detect RNA sequences from small cultured cell samples (Cheley and Anderson, 1984), the presence of viruses in human tissues (Achim *et al.*, 1994), asses intergeneric *Saccharum* × *Erianthus* hybrids (Besse *et al.*, 1997), detect barley yellow dwarf viruses in oat (Liu *et al*,. 2007), and detect *Potato spindle tuber viroid* in potato tubers (Owens and Diener, 1981; Vassilakos *et al.*, 2012). Here, dot blot hybridization assay is optimized as a routine tool to rapidly and routinely screen a large population of plants carrying small random chromosome introgressions from *Hordeum chilense* in the wheat background. This dot blot hybridization technique involves the direct application of a nucleic acid solution to a nylon membranes and detection with appropriate labeled probes. In addition, the technique was subsequently tested for other species combinations used in wheat breeding programs.

Materials and Methods

Plant material

The plant material used in this work included *Hordeum chilense* Roem. et Schult. and wheat lines (*T. aestivum* cv. Chinese Spring) carrying either one or two full copies of one *H. chilense* chromosome (monosomic or disomic *H. chilense* addition lines; 2n = 6x + 1 = 43 and 2n = 6x + 2 = 44, respectively), one or two copies of one telosomic *H. chilense* chromosome (monotelosomic and ditelosomic *H. chilense* addition lines; 2n = 6x + 1t = 42 + 1t and 2n = 6x + 2t = 42 + 2t, respectively) and wheat lines carrying small *H. chilense* chromosome introgressions (2n = 6x = 42). In addition, *Hordeum vulgare*, *Secale cereale* and *Agropyrum cristatum* species were also included in this

work. All the lines were kindly supplied by Dr. Steve Reader (JIC, Norwich, UK) except the wheat line carrying a small *H. chilense* introgression, which has been developed in our lab (Rey *et al.*, 2015, unpublished data).

Dot blot hybridization

Genomic DNA was extracted from frozen seedling leaves following Murray and Thomson, (1980) and modified by Hernández *et al.*, (2001).The integrity and concentration of the DNA was verified by electrophoresis in 1% agarose gel. DNA samples (200 ng) were blotted onto nylon membranes (Hybond N^+, Amersham International, Buckinghamshire, UK) and were prehybridized for 30 min at 75 ºC in 50% Formamide, 2× Saline-Sodium Citrate (SSC), 0,1% Sodium Dodecyl Sulfate (SDS), 2% Blocking Reagent (Roche Diagnostics, Meylan, France) for 30 min at 75 ºC with gentle shaking. The hybridization mixture, consisting in 50% Formamide, 2× SSC, 0.1% SDS, 600 ng of biotin-labeled genomic DNA as a probe (either *H. chilense*, *H. vulgare*, rye or *A. cristatum*, depending on the experiment), was added to the prehybridization buffer, and hybridization was conducted at 75 ºC for 8 min follow by an overnight incubation at 37ºC. After hybridization, membrane was incubated in a Petri dish with 100 mM Tris-Hcl (pH 7.5) and 15 mM NaCl (buffer 1) for 1 min, followed by incubation in blocking buffer (0.5% (w/v) (blocking reagent from Roche Diagnostics, Meylan, France diluted in 100 mM Tris-Hcl (pH 7.5) and 15 mM NaCl (buffer 2)) for 30 minutes, shaking gently. The membrane was incubated with antibiotin alkaline phosphatase (MACS, Bergish Gladbach, Germany) diluted in 1:100 in buffer 1 at 37ºC for 30 min, shaking gently. After the antibody incubation, the membrane was washed in buffer 1 for 15 min and then transferred to the detection buffer (100 mM Tris-HCl (pH 9.5); 100 mM NaCl; 50 mM $MgCl_2$) (buffer 3) for 2 min. Finally, the hybridization signals were developed by adding the detection reagents NBT (4-nitroblue tetrazolium chloride, 70 % dimethylformamide; Sigma, St. Louis, MO, USA) and BCIP (5-

bromo-4-chloro-2-indolylphosphate, 50 mg/ml in 70% dimethylformamide; Sigma, St. Louis, MO, USA) for 3 min in buffer 3 in the dark until the color was fully developed. Finally the membrane was washed in water and air-dried.

Genomic *in situ* hybridization

The presence of *H. chilense* genetic introgressions in wheat detected by the dot blot assay was confirmed by *in situ* hybridization. Total *H. chilense* genomic DNA was labeled by nick-translation with biotin-11-dUTP (Boehringer Mannheim Biochemicals, Germany) or digoxigenin-11-dUTP (Roche Applied Science, Indianapolis, IN, USA) and used as a probe. The *in situ* hybridization protocol was performed according to Prieto *et al.,* (2004). Biotin-labelled *H. chilense* DNA and digoxigenin-labelled *H. chilense* DNA were detected with antidigoxigenin-FITC (Roche Diagnostics, Meylan, France) and streptavidin-Cy3 conjugates (Sigma, St. Louis, MO, USA), respectively. Specifically, *H. chilense* DNA was always labeled with biotin-11-dUTP for dot blot hybridization. However, *H. chilense* DNA was labeled indistinctly with biotin-11-dUTP or digoxigenin-11-dUTP for *in situ* hybridization. Chromosomes were counterstained with DAPI (4', 6-diamidino-2-phenylindole) and mounted in Vectashield. Hybridization signals were visualized using a Nikon Eclipse 80i epifluorescence microscope. Images were captured with a Nikon CCD camera using the Nikon 3.0 software (Nikon Instruments Europe BV, Amstelveen, The Netherlands) and processed with Photoshop 4.0 software (Adobe Systems Inc., San Jose, California, USA).

Results

With the aim of detecting the minimum amount of genomic DNA which is detectable in a dot blot hybridization, different amounts of total genomic DNA from *H. chilense* were loaded in a membrane and were hybridize using biotin labeled total *H. chilense* genomic DNA as a probe. The dot blot assay showed

positive signals for all the slot blots except when 1 ng was loaded, revealing that the minimum amount of genomic DNA which is possible to detect by this technique was 5 ng (Figure 1).

In order to determine the sensitivity of the technique for detecting *H. chilense* genomic introgressions in the background of bread wheat, an experiment was carried out using different wheat lines carrying *H. chilense* chromosome introgressions of diverse size: disomic and monosomic *H. chilense* addition lines in bread wheat, monotelosomic and ditelosomic *H. chilense* addition lines and a wheat line carrying one copy of a distal fragment *H. chilense* chromosome segment (approximately 1/10 of the total chromosome length). To minimize unspecific hybridization signals due to common repetitive sequences between wheat and H. chilense, *Triticum aestivum* genomic DNA was used as blocking DNA. Results showed that it was possible to detect all the *H. chilense* genomic introgressions in the background of hexaploid wheat (Figure 2). Moreover, the size of the chromosome seems not to be limiting factor to identify wheat plants carrying *H. chilense* genomic introgressions that represents at least 1/10 of a wheat chromosome, among 42 wheat chromosomes, which means approximately a 1/420 dilution of the *H. chilense* DNA in the wheat background (Figure 2). The results obtained by the dot blot analysis were confirmed by *in situ* hybridization developed in somatic cells from the same wheat lines carrying one or two copies of a *H. chilense* chromosome, a monotelosomic or ditelosomic *H. chilense* chromosome or a distal small *H. chilense* chromosome segment in the wheat background (Figure 3).

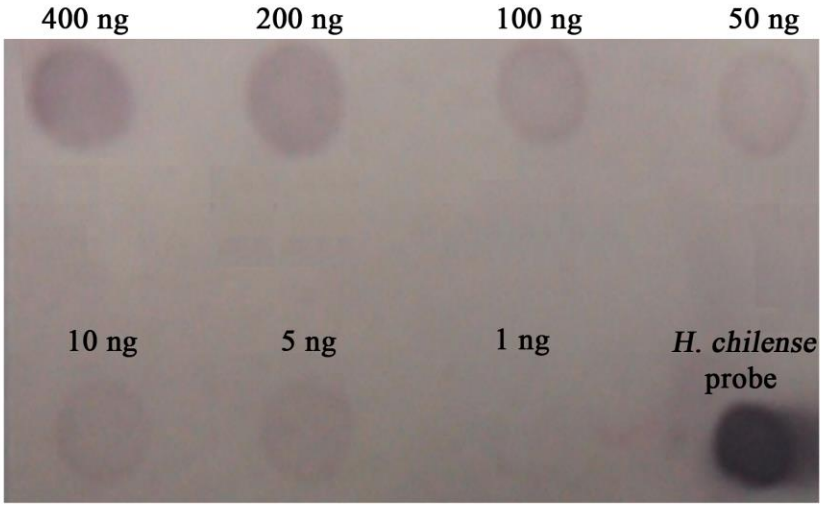

Figure 1. Dot blot hybridization to establish the minimum amount of *H. chilense* total genomic DNA detectable by this technique. Biotin-labeled *H. chilense* DNA was used as a probe. *Hordeum chilense* DNA amounts from 400 ng to 1 ng revealed the detection limit in 5 ng. C+: *H. chilense* DNA probe used as positive control.

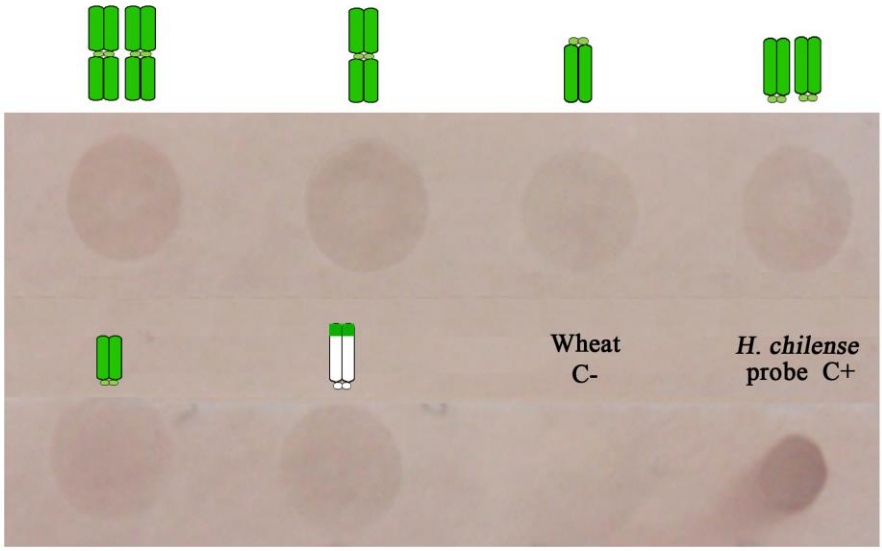

Figure 2. Dot blot hybridization using biotin-labeled *H. chilense* DNA as probe in *H. chilense* substitution lines in bread wheat. Slots: 4**H**^{ch} disomic addition line, (4**B**) 4**H**^{ch} monosomic substitution line, 7**H**^{ch}L monotelosomic addition line in wheat, 6**H**^{ch}S ditelosomic addition line in wheat, 6**H**^{ch}S monotelosomic addition line in wheat and wheat line carrying one copy of a distal 4**H**^{ch}L segment. C-: wheat DNA, negative control. C+: *H. chilense* total genomic DNA labeled, positive control. 200 ng of DNA was loaded per spot.

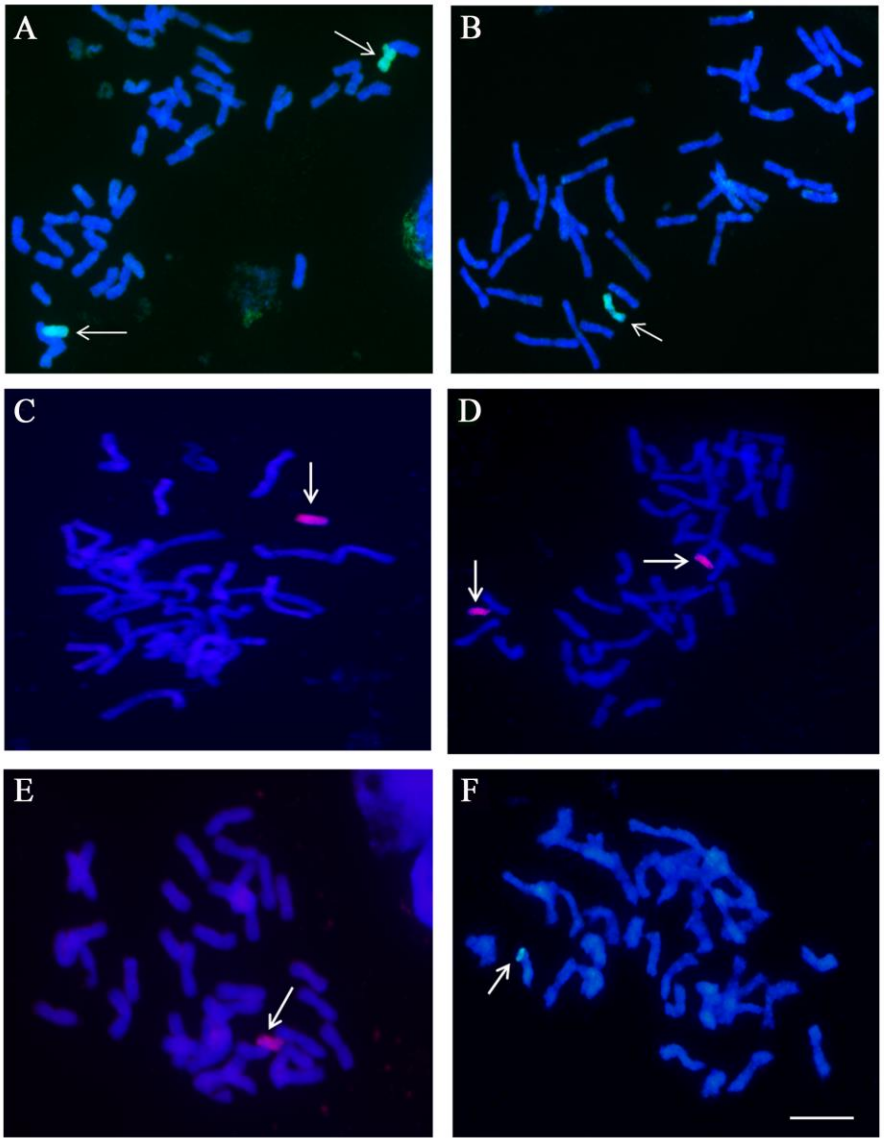

Figure 3. Genomic *in situ* hybridization using biotin and digoxigenin-labeled *H. chilense* genomic DNA probes in *H. chilense* introgression lines in bread wheat, detected either with rhodamine in red or fluorescein in green, respectively. A. 4**H**^{ch} disomic addition line, B. (4**B**) 4**H**^{ch} monosomic substitution line, C. 7**H**^{ch}L monotelosomic line, D. 6**H**^{ch}S ditelosomic line, E. 6**H**^{ch}S monotelosomic line and F. wheat line carrying one copy of a distal 4**H**^{ch}L segment on 4**D** chromosome. Bar = 10 µm.

Once it was demonstrated the success of the dot blot analysis to detect *H. chilense* chromosome introgressions in the wheat background, we carried out an experiment using 15 wheat plants of a segregating population from a genetic cross between (4**B**)4**H**$^{\mathbf{ch}}$ monosomic substitution line and *ph1b* mutant line to find those plants having *H. chilense* chromosome introgressions (Figure 4). Nine positive plants showed strong hybridization signals, suggesting that these plants carried a *H. chilense* introgression in the wheat background, either as a full copy or one or two chromosomes or smaller genomic introgressions as result of chromosome translocations or interspecific recombination between wheat and *H. chilense* chromosomes. Positive results were confirmed by *in situ* hybridization (data not shown). At the same time, negative results in the dot blot analysis always corresponded to wheat plants with no *H. chilense* chromosome introgressions (data not shown).

The reproducibility of the dot blot hybridization to detect *H. chilense* DNA into the background of bread wheat was also checked. A dot blot experiment was carried out loading on the membrane three replicates of five wheat plant of a segregating population from a genetic cross between (4**B**)4**H**$^{\mathbf{ch}}$ monosomic substitution line and *ph1b* mutant line. Two hundreds nanograms of total genomic DNA were loaded per sample. Positive signals were successfully detected in the three replicates from the same introgression line meanwhile negative signals were consistently negative in three replicates (Figure 5). Results showed that the dot blot assay is a consistent and reproducible method to detect *H. chilense* genomic introgressions in the wheat background.

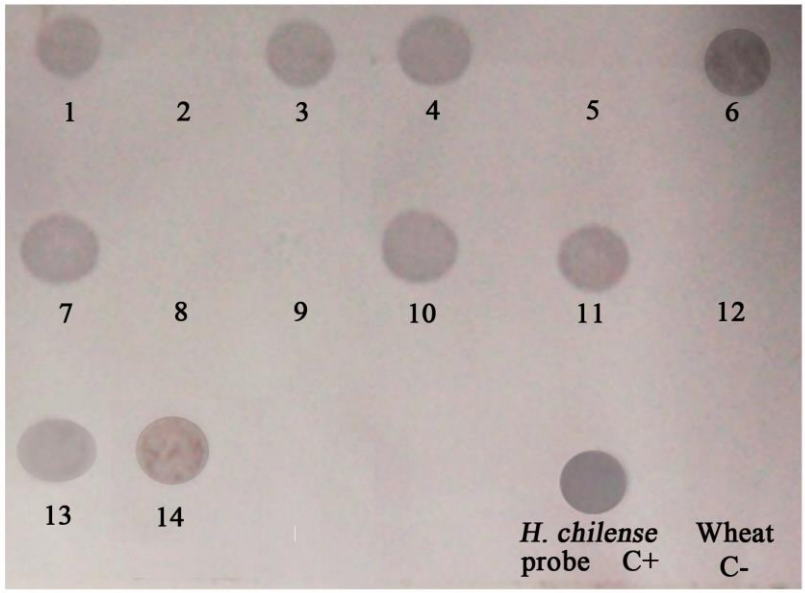

Figure 4. Dot blot hybridization in a wheat population from a genetic cross between (4B)4H^{ch} monosomic substitution line and *ph1b* mutant line to detect those plants carrying *H. chilense* genetic introgressions in the wheat background. Positive spots show presence of *H. chilense* and negative spots show the absence of *H. chilense* in bread wheat. C+: Biotin-labeled *H. chilense* DNA probe, positive control. C-: bread wheat, negative control. 200 ng of DNA was loaded per spot.

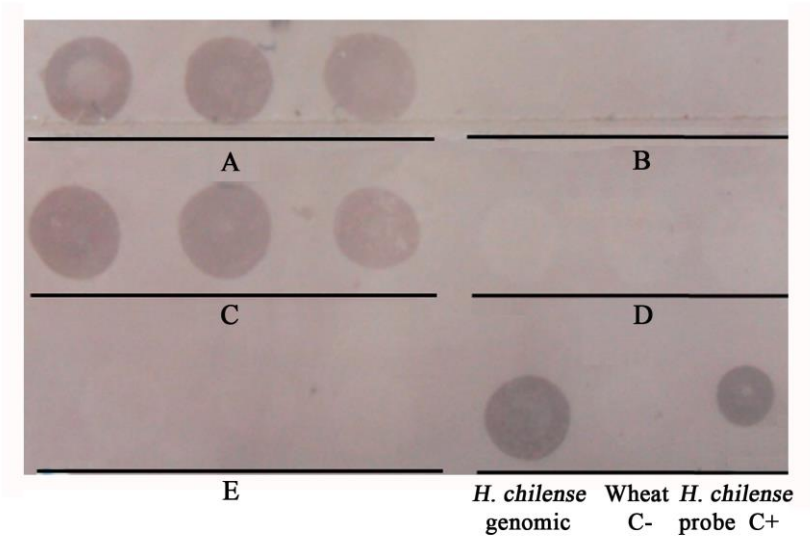

Figure 5. Reproducibility of the dot blot assay to detect *H. chilense* DNA genomic introgressions in a segregation population from a genetic cross between (4B)4H^{ch} monosomic substitution line and *ph1b* mutant line. Three replicates were loaded per plant, 200 ng of DNA in each one. A and C corresponded to wheat plants carrying a chromosome 4**H^{ch}** and B, D and E corresponded to wheat without any *H. chilense* chromosome introgression. Control spots: *H. chilense* DNA, C+: Biotin-labeled *H. chilense* DNA probe, positive control,
C-: wheat DNA, negative control.

Dot blot assay was also validated to screen other alien genetic introgressions in a wheat population. Plants carrying *H. vulgare* chromosome introgressions were detected in a dot blot assay using total *H. vulgare* genomic DNA as a probe (Figure 6a). Similarly this approach allowed the screening and detection of *Agropyrum cristatum* and *Secale cereale* genomic DNA in the wheat background (Figure 6b, c, respectively). In these cases, the positive controls were biotine- *A. cristatum* DNA and biotin- *S. cereale* DNA, respectively.

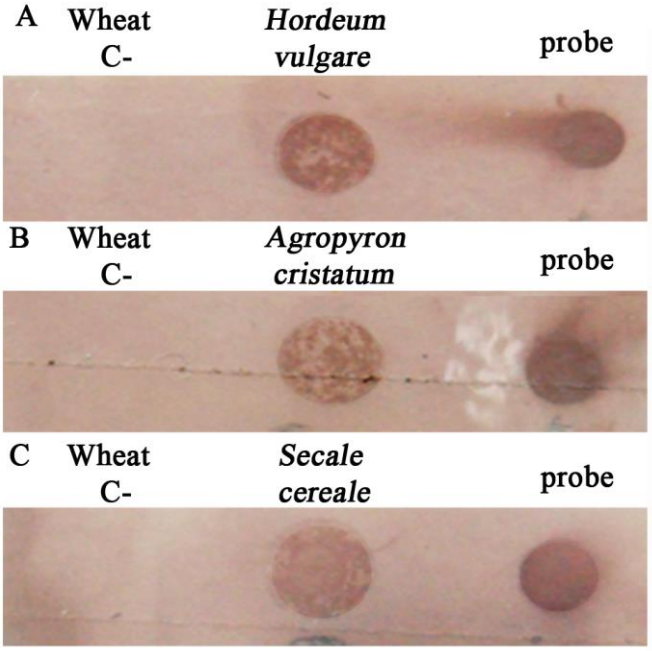

Figure 6. Dot blot hybridization to detect A. *H. vulgare* chromosome, B. *Agropyron cristatum* and C. *Secale cereale* chromosome introgressions in the wheat background. C-: wheat DNA, negative control, biotin-labeled *Hordeum vulgare*, *Agropyron cristatum* and *Secale cereale* DNA labeled probes were used as positive controls (probe). Two hundred ngs of DNA were loaded per spot.

Discussion

We have demonstrated that dot blot hybridization assay can be used as a rapid and routine tool for the screening of a large population of plants from genetic crosses carrying small random chromosome introgressions from relative species in the wheat background. The optimization of concentration of DNA blocking is a key step due to the presence of repeat sequences in cereals (Flavell *et al.*, 1977). DNA was labeled indistinctly with biotin-11-dUTP or digoxigenin-11-dUTP because the intensity of the signal was very similar in *in situ* hybridization experiments. However, in the dot blot hybridization assay, the signal was much weaker with digoxigenin than with biotine (data not shown)

and for this reason in all experiments biotine was used to label the DNA probes as a routine.

Nowadays, there are several techniques available for the detection of alien introgression in the wheat background, including C-banding, molecular markers or *in situ* hybridization. Although molecular markers and *in situ* hybridization are useful tools to select the plant material of interest (Forster *et al.,* 2000; Prieto *et al.,* 2001), both techniques have several disadvantages such as expensive and time-consuming. In one hand, the use of molecular markers is very limited when small fragments of related species are achieved in bread wheat. Genetic maps of relative species are not saturated either, what sometimes difficult the screening of alien genetic introgressions from relative species based only in molecular markers. In addition, the use of molecular markers is based on the previous knowledge of the exact chromosome introgression but can be useless when the chromosome or chromosome segment from the relative species involved in recombination are not well characterized or chromosome introgressions from the relative species occurred randomly in the wheat background. Developing PCR-bases molecular markers for a specific alien chromosome can accelerate selection in a breeding program. For example, 607 EST-derived and 82 SSR primers pairs were used for amplification and 32 were specific for 4**V** (*Haynaldia villosa)*. However, only five specific markers could be detected in the WYMV-resistant terminal translocation line NAU421 with the shortest introduced 4**V**S fragment (Zhao *et al*., 2013). Most SSR markers are genome-specific and their transferability across related species is low (Mullan *et al.*, 2005). Qi et al., 2007 had serious problems to detect recombinants because there were not enough molecular markers to determine the presence of *Th. intermedium* in wheat. They used more than 16 000 EST loci to define the *Th. intermedium* specific chromosome regions in wheat. Unfortunately, only nine STS markers were polymorphics between the *Th. intermedium* and wheat. On the other hand, the *in situ* hybridization is the most efficient and most accurate

technique for allocating the breakpoints and estimating for allocating chromatin in the translocation chromosomes (Le *et al.,* 1989; Schwarzacher *et al.,* 1989; Jiang and Gill, 1994) but requires intact nuclei and samples must be processed within a short amount of time. In addition, it is an expensive and technically more complicated methodology which makes *the in situ* hybridization not valid to screen a wide number of plants in a segregating population. It is much more convenient and efficient to analyze those plants which were positive using a low cost and quick technique which does not require high qualification. Another method to detect alien introgression in wheat has been the C-banding procedure. Lukaszewski in 2000 used this procedure to screen 20234 progeny but only recovered 139 primary recombinants for the rye 1**R**S and wheat 1**S** short arms.

The dot blot hybridization can be very useful in wheat breeding programs when the manipulation of chromosome associations between relative species and wheat is carried out in the absence of the *Ph1* locus (Sears, 1977). The use of the *ph1b* mutant has been widely used to transfer useful genes from wild relatives into wheat for the improvement of resistances to biotic and abiotic stresses (Friebe *et al.,* 1996; Xin *et al.,* 2001; Mullan *et al.,* 2009). The use of only molecular markers or *in situ* hybridization would be high cost and difficult to locate small alien genetic introgressions because these occur randomly. Thus, among the methods that allow the detection of alien introgressions in the wheat background, dot blot hybridization facilitates the analysis being very fast and reliable.

Although cereals genomes contain a very high proportion (>75%) of repeated sequences, which have been extensively investigated in the genus *Hordeum* (Flavell and Smith, 1976; Bedbrook *et al.,* 1980) the high sensitivity and reliability of the molecular hybridization assay described here introduce a basic method to detect the presence of chromosome introgressions from relative species in the background of wheat. Therefore, this technique can be, useful in

plant breeding programs, when it is necessary to screen wide segregating populations. Moreover, this protocol has been revealed suitable to detect other genomic introgressions from species such as *H. vulgare*, *S. cereale* and *A. cristatum* in the wheat background.

To summarize, dot blot hybridization can be a low cost and short time consuming assay to analyze large number samples in a genetic population carrying alien genetic introgressions, although this technique must be complement with the use of *in situ* hybridization to fully characterize introgressions lines.

Chapter VI

Conclusions/Conclusiones

1. The observations on replication in the presence and in the absence of the *Ph1* locus showed that DNA replication during early meiosis lasts longer and finishes later when the *Ph1* locus was present. The role of the *Ph1* locus in replication suggested a solid connection between DNA replication and chromosome associations at the onset of meiosis in a polyploid like wheat.

2. *Hordeum chilense* and *H. vulgare* genetic introgressions were obtained in the background of the *ph1b* mutant. The use of the *ph1b* mutant did induce a low, but significant level of chromosome pairing and recombination between wheat and *Hordeum* sp. chromosomes. Homoeologous recombination between *Hordeum* sp. and wheat chromosomes did only depend on the absence of the *Ph1* locus.

3. New wheat-*H. chilense* translocation lines for both 7$\mathbf{H}^{\mathbf{ch}}\alpha$ and 7$\mathbf{H}^{\mathbf{ch}}\beta$ *H. chilense* chromosome arms were development in wheat in the background of the *ph1b* mutant. Both 7$\mathbf{H}^{\mathbf{ch}}\alpha$·7**A**L and 7**A**S·7$\mathbf{H}^{\mathbf{ch}}\beta$ disomic translocation lines were enriched in carotenoid content. A proteomic analysis confirmed that the presence of chromosome 7$\mathbf{H}^{\mathbf{ch}}$ introgressions did not alter the proteomic profile of the wheat flour. HSPs and a 14-3-3-like protein play a key role in the enhancement of carotenoid accumulation in seed. The *Psy1* gene involved in the carotenoid biosynthetic pathway was also cytogenetically mapped on the 7$\mathbf{H}^{\mathbf{ch}}\alpha$ chromosome arm.

4. An efficient and user-friendly method for the screening of small random genomic introgressions from *Hordeum chilense* in the wheat germplasm has been developed. This technique allowed a rapid and reliable screening for detecting small random *H. chilense* genomic introgressions introduced during a breeding program in the background of bread wheat, saving time and money. The robustness of the technique was confirmed by *in situ* hybridization. The

methodology was revealed to be also suitable to detect genetic introgressions from other wheat relative species such as *Hordeum vulgare*, *Secale cereale* and *Agropyron cristatum* in the wheat background.

1. El estudio de la replicación en presencia y en ausencia del locus *Ph1* demostró que la replicación es más larga y termina después cuando el locus *Ph1* locus está presente. El efecto del locus *Ph1* en la replicación sugiere una conexión sólida entre la replicación del ADN y las asociaciones cromosómicas que ocurren al inicio de la meiosis en un poliploide como el trigo.

2. Se han obtenido introgresiones genéticas de *Hordeum* chilense y *H. vulgare* en el fondo genético del mutante *ph1b*. El uso del mutante *ph1b* provocó un bajo pero significativo nivel de apareamiento entre los cromosomas de trigo y ambas especies de *Hordeum.* Se ha observado que la recombinación homoeóloga entre los cromosomas de ambas especies de *Hordeum* y los cromosomas de trigo se debe solo a la ausencia del locus *Ph1*.

3. Se han desarrollado nuevas líneas de translocación para ambos brazos cromosómicos del cromosoma 7$\mathbf{H}^{ch}$ de *H. chilense* en el fondo genético del mutante *ph1b*. Ambas líneas disómicas de translocación (7$\mathbf{H}^{ch}\alpha$·7**A**L and 7**A**S·7$\mathbf{H}^{ch}\beta$) mostraron un elevado enriquecimiento en carotenoides. Se ha confirmado mediante un análisis proteómico que la presencia del cromosoma 7$\mathbf{H}^{ch}$ no altera el perfil proteómico de la harina de trigo. Además el estudio proteómico reveló que las proteínas HSPs y 14-3-3-like protein podrían desempeñar un papel en el enriquecimiento del grano en carotenoides. Se mapeó mediante hibridación *in situ* el gen *Psy1,* el cual está involucrado en la síntesis de carotenoides, en el brazo cromosómico 7$\mathbf{H}^{ch}\alpha$.

4. Se ha optimizado un método rápido y eficiente para detectar pequeñas introgresiones genéticas de *Hordeum chilense* en el fondo genético del trigo. La robustez y fiabilidad de la técnica se confirmó mediante hibridación *in situ*. Además el método puede ser extrapolado a la detección de introgresiones

genéticas de otras especies cercanas al trigo como *Hordeum vulgare*, *Secale cereale* y *Agropyron cristatum* en el fondo genético del trigo harinero.

Chapter VII

References

Abranches R, Beven AF, Aragón-Alcaide L, Shaw P (1998) Transcription sites are not correlated with chromosome domains in wheat nuclei. The Journal of Cell Biology 143: 5-12

Achim R, Anton G, Stoian M, Repanovici B (1994) Nucleic acid dot blot hybridization with fragmented digoxigenin labelled probes in human papillomavirus DNA screening. Revue Romaine de Virologie 45: 109-113

Alexandrow MG, Hamlin JL (2005) Chromatin decondensation in S-phase involves recruitment of Cdk2 by Cdc45 and histone H1 phosphorylation. The Journal of Cell Biology 168: 875-886

Al-Kaff N, Knight E, Bertin I, Foote TN, Hart N, Griffiths S, Moore G (2008) Detailed dissection of the chromosome region containing the *Ph1* locus in wheat *Triticum aestivum*: with deletion mutants and expression profiling. Annals of Botany 101: 863-872

Altenbach SB, Vensel WH, Dupont FM (2011) The spectrum of low molecular weight alpha-amylase/protease inhibitor genes expressed in the US bread wheat cultivar Butte 86. BMC Research Notes 4: 242

A**lvarez JB, Martín LM, Martín A (1998)** Chromosomal localization of genes for carotenoid pigments using addition lines of *Hordeum chilense* in wheat. Plant Breeding 117: 287-289

Anamthawat-Johnsson K, Heslop-Harrison JS (1990) Centromeres, telomeres and chromatin in the interphase nucleus of cereals. Caryologia 43: 205-213

Aragón-Alcaide L, Miller T, Schwarzacher T, Reader S, Moore G (1996) A cereal centromeric sequence. Chromosoma 105: 261-268

Aragón-Alcaide L, Reader S, Beven A, Shaw P, Miller T, Moore G (1997) Association of homologous chromosomes during floral development. Current Biology 7: 905-908

Aragón-Alcaide L, Reader S, Miller T, Moore G (1997a) Centromeric behaviour in wheat with high and low homoeologous chromosomal pairing. Chromosoma 106: 327-333

Atienza SG, Ramírez CM, Hernández P, Martín A (2004) Chromosomal location of genes for carotenoid pigments in *Hordeum chilense*. Plant breeding 123: 303-304

Atienza SG, Ávila CM, Martín A (2007) The development of a PCR-based marker for *PSY1* from *Hordeum chilense*, a candidate gene for carotenoid content accumulation in tritordeum seeds. Australian Journal Agriculture Research 58: 767-773

Bass HWO, Riera-Lizarazu EV, Ananiev SJ, Bordoli HW, Rines HW, Phillips RL, Sedat JW, Agard DA, Cande WZ (2000) Evidence for the coincident initiation of homolog pairing and synapsis during the telomere-clustering (bouquet) stage of meiotic prophase. The Journal of Cell Biology 113: 1033-1042

Bedbrook JR, Jones J, O´Dell M, Thompson RD, Flavekk RB (1980) A molecular description of telomeric heterochromatin in Secale species. Cell 19: 545-560

Benavente E, Fernández-Calvín B, Orellana J (1996) Relationship between the levels of wheat-rye metaphase I chromosomal pairing and recombination revealed by GISH. Chromosome 105: 92-96

Bennett MD, Smith JB (1972) The effect of polyploidy on meiotic duration and pollen development in cereal anthers. Proceedings of the Royal Society of London 181: 81–107

Besse P, McIntyre CL, Burner DM, Almeida CG (1997) Using genomic slot dot hybridization to assess intergeneric *Saccharum* × *Erianthus* hybrids (Andropogoneae – Saccharinae). Genome 40: 428-432

Bino RJ, De Vries JN, Kraak HL,Van Pijlen JG (1992) Flow cytometric determination of nuclear replication stages in tomato seeds during priming and germination. Annals of Botany 69: 231-236

Bino RJ, Lanteri S, Verhoeven HA, Kraak HL (1993) Flow cytometric determination of nuclear replication stages in seed tissues. Annals of Botany 72: 181-187

Borovkova IG, Steffenson BJ, Jin Y, Rasmussen JB, Kilian A, Kleinhofs A, Rossnagel BG, Kao Kn (1995) Identification of molecular markers linked to the stem rust resistance gene *rpg4* in barley. Phytopathology 85: 181–185

Brito G, Lopes T, Loureiro J, Rodriguez E, Santos C (2010) Assessment of genetic stability of two micropropagated wild olive species using flow cytometry and microsatellite markers. Trends in Ecology & Evolution 24: 723-732

Cabrera A, Friebe B, Jiang J, Gill BS (1995) Characterization of *Hordeum chilense* chromosomes by C-banding and *in situ* hybridization using highly repeated DNA probes. Genome 38: 435-442

Calderón MC, Ramírez MC, Martín A, Prieto P (2012) Development of *Hordeum chilense* 4**H**$^{\mathbf{ch}}$ introgression lines in durum wheat: a tool for breeders and complex trait analysis. Plant Breeding 131: 733-738

Calderón MC, Rey MD, Cabrera A, Prieto P (2014) The subtelomeric region is important for chromosome recognition and pairing during meiosis. Scientific Reports 4: 6488

Carmona L, Zacarías L, Rogrigo MJ (2012) Stimulation of coloration and carotenoid biosynthesis during postharvest storage of 'Navelina' orange fruit at 12ºC. Postharvest Biology and Technology 74: 108-117

Carvalho LJ, Lippolis J, Chen S, Batista de Souza CR, Vieira EA, Anderson JV (2012) Characterization of carotenoid-protein complexes and gene expression analysis associated with carotenoid sequestration in pigmented cassava (*Manihot Esculenta* Crantz) storage root. The Open Biochemistry Journal 6: 116-130

Castro AM, Ramos S, Vasicek A, Worland A, Giménez D, Clúa AA, Suárez E (2011) Identification of wheat chromosomes involved with different types of resistance against greenbug (*Schizaphis graminum*, Rond.) and the Russian wheat aphid (*Diuraphis noxia*, Mordvilko). Euphytica 118: 321-330

Ceoloni C, del Signore G, Ercoli L, Donini P (1992) Locating the alien chromatin segment in common wheat-*Aegilops longissima* mildew resistant transfers. Hereditas 116: 239-245

Cha RS, Weiner BM, Keeney S, Dekker J, Kleckner N (2000) Progression of meiotic DNA replication is modulated by interchromosomal interaction proteins, negatively by Spo11p and positively by Rec8p. Genes & Development 14: 493–503

Chantret N, Salse J, Sabot F, Rahman S, Bellec A, Laubin B, Duboir I, Dossat C, Sourdille P, Joudrier P, Gautier MF, Cattolico L, Beckert M, Aubourg S, Weissenbach J, Caboche M, Bernard M, Leroy P, Chalhoub B (2005) Molecular basis of evolutionary events that shaped the hardness

locus in diploid and polyploid wheat species (*Triticum* and *Aegilops*). Plant Cell 17: 1033-1045

Chapman V, Riley R (1955) The disomic addition of rye chromosome II to wheat. Nature 175: 1091

Cheley S, Anderson R (1984) A reproducible microanalytical method for the detection of specific RNA sequences by dot-blot hybridization. Analytical Biochemistry 137: 15-19

Colas I, Shaw P, Prieto P, Wanous M, Spielmeyer W, Mago R, Moore G (2008) Effective chromosome pairing requires chromatin remodeling at the onset of meiosis. Proceedings of the National Academy of Sciences 105: 6075-6080

Collado-Romero M, Alós E, Prieto P (2014) Unravelling the proteomic profile of rice meiocytes during early meiosis. Frontiers in Plant Science 5: 356

Collins I, Newlon CS (1994) Chromosomal DNA replication initiates at the same origins in meiosis and mitosis. Molecular and Cellular Biology 14: 3524–3534

Cowan CR, Carlton PM, Cande WZ (2001) The polar arrangement of telomeres in interphase and meiosis: Rabl organization and the bouquet. Plant Physiology 125: 532-538

Cox AV, Bennett ST, Parokonny AS, Kenton A, Callimassia MA, Bennet D (1993) Comparison of plant telomere localizations using a PCR-generated synthetic probe. Annals of Botany 72: 239-247

Dawe KR, Sedat JW, Agard DA, Cande ZW (1994) Meiotic chromosome pairing in maize is associated with a novel chromatin organization. Cell 76: 901-912

De Angelis M, Minervini F, Caputo L, Cassone A, Coda R, Calasso MP, Divella F, Gobbetti M (2008) Proteomic analysis by two-dimensional gel electrophoresis and starch characterization of *Triticum turgidum* L. var. durum cultivars for pasta making. Journal of Agricultural and Food Chemistry 56: 8619-28

Dolezel J (1998) Flow cytometry, its application and potential for plant breeding. In: Lelley T. (ed.) Current topics in plant cytogenetics related to plant improvement. Universitätsverlag, Vienna, Austria, pp. 80-90

Dolezel J, Bartos J (2005) Plant DNA flow cytometry and estimation of nuclear genome size. Annals of Botany 95: 99-110

Drouin R, Lemieux N, Richer CL (1991) Chromosome condensation from prophase to late metaphase: relationship to chromosome bands and their replication time. Cytogenetics and Cell Genetics 57: 91-99

Duveiller E, Singh RP, Nicol JM (2007) The challenges of maintaining wheat productivity: pests, diseases, and potential epidemics. Euphytica 157: 417-430

Eilam T, Anikster Y, Millet E, Manisterski J, Sagi-Assif O, Feldman M (2007) Genome size and genome evolution in diploid Triticeae species. Genome 50: 1029-1037

Endo TR (1988) Induction of chromosomal structural changes by a chromosome of *Aegilops cylindrical* L. in common wheat. Journal of Heredity 79: 366-370

Endo TR (1990) Gametocidal chromosomes and their induction of chromosome mutations in wheat. The Japanese Journal of Genetics 65: 135-152

Endo TR, Gill BS (1996) The deletion stocks of common wheat. Journal of Heredity 87: 295-307

Endo TR, Shi F, Tsvetkov KS (1998) Genetic induction of chromosomal structural changes of alien chromosomes in common wheat. In: A.E. Slinkard (Ed.), Proc 9th Int Wheat Genet Symp, Saskatoon, Canada 2: 40-43

Endo TR (2007) The gametocidal chromosome as a tool for chromosome manipulation in wheat. Chromosome Research 15: 67-75

Farooq S, Shah TM, Iqbal N (1990) Variation in crossability among intergeneric hybrids of wheat and salt tolerant accessions of three *Aegilops* species. Cereal Research Communications 18: 335-338

Farré G, Sanahuja G, Naqvi S, Bai C, Capell T, Zhu C, Christou P (2010) Travel advice on the road to carotenoids in plants. Plant Science 179: 28-48

Feuillet C, Lagridge P, Waugh R (2008) Cereal breeding takes a walk on the wild side. Trends in Genetics 24: 24-32

Flavell RB, Smith DB (1976) Nucleotide sequence organization in the wheat genome. Heredity 37: 231-252

Flavell RB, Rimpau J, Smith DB (1977) Repeated sequence DNA relationships in four cereal genomes. Chromosoma 63: 205-222

Forsburg SL, Hodson JA (2000) Mitotic replication initiation proteins are not required for pre-meiotic S phase. Nature Genetics 25: 263-268

Forsburg SL (2002) Only connect: linking meiotic DNA replication at chromosome dynamics. Molecular Cell 9: 703-711

Forster BP, Phillips MS, Miller TE, Baird E, Powell W (1990) Chromosome location of genes controlling tolerance to salt (NaCl) and vigour in *Hordeum vulgare* and *H. chilense.* Heredity 65: 99-107

Forster BP, Ellis RP, Thomas WTB, Newton AC, Tuberosa R, This D, El-Enein RA, Bahri MH, Salem MB (2000) The development and application of molecular markers for abiotic stress tolerance in barley. Journal of Experimental Bonaty 51: 19-27

Friebe B, Jiang J, Raupp WJ, McIntosh RA, Gill BS (1996) Characterization of wheat-alien translocations conferring resistance to diseases and pests: current status. Euphytica 91:59–87

Fung PK, Krushkal J, Weathers PJ (2010) Computational analysis of the evolution of 1-deoxy-D-xylulose-5-phosphate Reductoisomerase, an important enzyme in plant terpene biosynthesis. Chemistry and Biodiversity 7: 1098-1110

Gilbert DM (2001) Making sense of eukaryotic DNA replication origins. Science 294: 96-100

Gilbert DM (2002) Replication timing and transcriptional control: beyond cause and effect. Current Opinion in Cell Biology 14: 377-383

Gill BS, Raupp WJ (1987) Direct genetic transfers from *Aegilops squarrosa* L. to hexaploid wheat. Crop Science 27: 445-450

Giroux MJ, Morris CF (1998) Wheat grain hardness results from highly conserved mutations in the friabilin components puroindoline a and b. Proceedings of the National Academy of Sciences 95: 6262-6266

Grallert B, Sipiczki M (1991) Common genes and pathways in the regulation of the mitotic and meiotic cell cycles of *Schizosaccharomyces pombe*. Current Genetics 20: 199–204

Graner A, Jahoor A, Schondelmaier J, Siedler H, Pillen K, Fischbeck G, Wenzel G, Herrmann RG (1991) Construction of an RFLP map of barley. Theoretical and Applied Genetics 83: 250–256

Greer E, Martin AC, Pendle A, Colas I, Jones AME, Moore G, Show P (2012) The *Ph1* locus suppresses cdk2-type activity during premeiosis and meiosis in wheat. The Plant Cell 24: 152-162

Griffiths S, Sharp R, Foote TN, Bertin I, Wanous M, Reader S, Colas I, Moore G (2006) Molecular characterization of *Ph1* as a major chromosome pairing locus in polyploid wheat. Nature 439: 749-752

Gu YQ, Coleman-Derr D, Kong X, Anderson OD (2004) Rapid genome evolution revealed by comparative sequence analysis of orthologous regions from four Triticeae genomes. Plant physiology 135: 459-470

Gu YQ, Salse J, Coleman-Derr D, Dupin A, Crossman C, Lazo GR, Belcram H, Ravel C, Charmet G, Charles M, Anderson OB, Chalhoub B (2006) Types and rates of sequences evolution at the high molecular-weight glutenin locus in hexaploid wheat and its ancestral genomes. Genetics 174: 1493-1504

Gutierrez C (1998) The retinoblastoma pathway in plant cell cycle and development. Current Opinion in Plant Biology 1: 492–497

Hagras AAA, Masahiro K, Tanaka K, Sato K, Tsujimoto H (2005) Genomic differentiation of *Hordeum chilense* from *H. vulgar*e as revealed by repetitive and EST sequences. Genes & Genetic Systems 80: 147-159.

Halvorsen BL, Holte K, Myhrstad MCW, Barikmo I, Hvattum E, Remberg SF, Wold AB, Haffner K, Baugerod H, Andersen LF, Moskaug O, Jacobs Jr DR, Blomhoff R (2002) A systematic screening of total antioxidants in dietary plants. Journal of Nutrition 132: 461-471

Harper JW, Adams PD (2001) Cyclin-Dependent Kinases. Chemical Reviews 101: 2511-2526

Harper L, Golubovskaya I, Cande WZ (2004) A bouquet of chromosomes. Journal of Cell Science 117: 4025-4032

Hentschel V, Katja K, Hollmann J, Lindhauer MG, Böhm V, Bitsch R (2002) Spectrophotometric determination of yellow pigment content and evaluation of carotenoids by high-performance liquid chromatography in durum wheat grain. Journal of Agricultural and Food Chemistry 50: 6663–6668

Hernández P, Dorado G, Prieto P, Jiménez MJ, Ramírez MC. Laurie DA, Snape JW, Martín A (2000) A core genetic map of *Hordeum chilense* and comparisons with maps of barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*). Theoretical and Applied Genetics 102: 1259-1264

Holm PB (1977) The premeiotic DNA replication of euchromatin and heterochromatin in *Lilium longiflorum* (Thunb.). Carlsberg research communications 42: 249–281

Hotta Y, Stern H (1961) Transient phosphorylation of deoxyribosides and regulation of deoxyribonucleic acid synthesis. The Journal of Biophysical and Biochemical Cytology 11: 311-319

Howitt CA, Cavanagh CR, Bowerman AF, Cazzonelli C, Rampling L, Mimica JL, Pogson BJ (2009) Alternative splicing, activation of cryptic

exons and amino acid substitutions in carotenoid biosynthetic genes are associated with lutein accumulation in wheat endosperm. Functional and Integrative Genomics 9: 363-376

Huntley RP, Murray JAH (1999) The plant cell cycle. Current Opinions in Plant Biology 2: 440-446

Islam AKMR, Shepherd KW, Sparrow DHB (1978) Production and characterization of wheat-barley addition lines. Proceedings of the 5th International Wheat genetics Symposium. Science Publishers Inc, India, 356-371

Islam AKMR, Shepherd KW, Sparrow DHB (1981) Isolation and characterization of euplasmic wheat-barley chromosome addition lines. Heredity 46: 161-174

Islam AKMR, Shepherd KW (1988) Induced pairing between wheat and barley chromosomes. In: Miller TE, Koebner RMD, eds. Proceedings of the 7th International Wheat Genet Symposium, Cambridge, England, 309-314

Islam AKMR, Shepherd KW (1990) Incorporation of barley chromosomes into wheat. In: Y.P.S. Bajaj (Ed), Biotechnology in Agriculture and Forestry, Vol 13 Wheat. Pp. 128-151. SpringerVerlag Publishers, Berlin

Islam AKMR, Shepherd KW (2000) Isolation of a fertile wheat-barley addition line carrying the entire barley chromosome 1H. Euphytica 111: 145-149

Jasencakova Z, Meister A, Schubert I (2001) Chromatin organization and its relation to replication and histone acetylation during the cell cycle in barley. Chromosoma 110: 83-92

Jiang J, Gill BS (1994) Nonisotopic *in situ* hybridization and plant genome mapping: the first 10 years. Genome 37: 717-725

Jiang J, Friebe B, Gill BS (1994) Recent advances in alien gene transfer in wheat. Euphytica 73: 199-212

Jordan P (2006) Initiation of homologous chromosome pairing during meiosis. Biochemical Society Transactions 34: 545-549

Kaneko S, Nagamine T, Yamada T (1995) Esterification of endosperm lutein with fatty acids during the storage of wheat seeds. Bioscience, Biotechnology, and Biochemistry 59: 1–4

Karlova R, Rosin FM, Busscher-Lange J, Parapunova V, Do PT, Fernie AR, Fraser PB, Baxter C, Angenent GC, de Maagd RA (2011) Transcriptome and metabolite profiling show that APETALA2a is a major regulator of tomato fruit ripening. Plant Cell 23: 923-941

Khan IA (1999) Detection of wheat-alien recombinant chromosomes using co-dominant DNA markers. Annals of Applied Biology 135: 579-583

Kleinhofs A, Kilian A, Maroof MAS, Biyashev RM, Hayes PM, Chen FQ, Lapitan N, Fenwick A, Blake TK, Kanazin V, Ananiev E, Dahleen L, Kudrna D, Bollinger J, Knapp SJ, Liu B, Sorrells M, Heun M, Franckowiak JD, Hoffman D, Skadsen R, Steffenson BJ (1993) A molecular, isozyme and morphological map of the barley (*Hordeum vulgare*) genome. Theoretical and Applied Genetics 86: 705–712

Koebner RMD, Shepherd KW (1986) Controlled introgression to wheat of genes from rye chromosome arm 1RS by induction of allosyndesis. Theoretical and Applied Genetics 73: 197-208

Koebner RMD, Shepherd KW (1987) Allosyndetic recombination between a chromosome of *Aegilops umbellulata* and wheat chromosomes. Heredity 59: 33-45

Komatsuda T, Mano Y (2002) Molecular mapping of the intermedium spike-c (*int-c*) and non-brittle rachis 1 (*btr1*) loci in barley (*Hordeum vulgare* L.) Theoretical and Applied Genetics 105: 85-90

Kotogány E, Dudits D, Horváth GV, Ayaydin F (2010) A rapid and robust assay for detection of S-phase cell cycle progression in plant cells and tissues by using ethynyl deoxyuridine. Plant methods 6: 5

Larré C, Lupi R, Gombaud G, Brossard C, Branlard G, Moneret-Vautrin DA, Rogniaux H, Denery-Papini S (2011) Assessment of allergenicity of diploid and hexaploid wheat genotypes: identification of allergens in the albumin/globulin fraction. Journal of Proteomics 74: 1279-1289

Le HT, Armstrong KC, Miki B (1989) Detection of rye DNA in wheat-rye hybrids and wheat translocation stocks using total genomic DNA as a probe. Plant Molecular Biology Reporter 7: 150–158

Lepage M, Sims RPA (1968) Carotenoids of wheat flour: Their identification and composition. Cereal Chemistry 45: 600-604

Leus L, Van Laere K, Dewitte A, Van Huylenbroeck J (2009) Flow cytometry for plant breeding. Acta horticulture 836: 221-226

Li F, Vallabhaneni R, Wurtzel ET (2008) PSY3, a new member of the phytoene synthase gene family conserved in the Poaceae and regulator of abiotic stress-induced root carotenogenesis. Plant Physiology 146: 1333-1345

Lima-de-Faria A, German J, Ghatnekar M, McGovern J, Anderson L (1966) DNA synthesis in the meiotic chromosomes of man. A preliminary report. Hereditas 56: 398-399

Lima-de-Faria A, German J, Ghatnekar M, McGovern J, Anderson L (1968) In vitro labelling of human meiotic chromosomes with H^3-Thymidine. Hereditas 60: 249-261

Liu ZW, Biyashev RM, Saghai Maroof MA (1996) Development of simple sequence repeat DNA markers and their integration into a barley linkage map. Theoretical and Applied Genetics 93: 869-876

Liu Y, Sun B, Wang X, Zheng C, Zhou G (2007) Three digoxigenin-labeled cDNA probes for specific detection of the natural population of Barley yellow dwarf viruses in China by dot blot hybridization. Journal of Virological Methods 145: 22-29

Liu W, Jin Y, Rouse M, Friebe B, Gill B, Pumphrey MO (2011) Development and characterization of wheat-*Ae. searsii* robertsonian translocations and a recombinant chromosome conferring resistance to stem rust. Theoretical and Applied Genetics 122: 1537-1545

Lukaszewki AJ (2000) Manipulation of the 1RS.1BL translocation in wheat by induced homoeologous recombination. Crop Science 40: 216-225

Luo MC, Yang ZL, You FM, Kawahara T, Waines JG, Dvorak J (2007) The structure of wild and domesticated emmer wheat populations, gene flow between them, and the site of emmer domestication. Theoretical and Applied Genetics 114: 1312-1314

Maestra B, De Jong JH, Shepherd K, Naranjo T (2002) Chromosome arrangement and behaviour of two rye telosomes at the onset of meiosis in

disomic wheat-5RL addition lines with and without the *Ph1* locus. Chromosome Research 10: 665-667

Marshall WF, Dernburg AF, Harmon B, Agard DA, Sedat JW (1996) Specific interactions of chromatin with the nuclear envelope: positional determination within the nucleus in *Drosophila melanogaster*. Molecular Biology of the Cell 7: 825-842

Martín A, Chapman V (1977) A hybrid between *Hordeum chilense* and *Triticum aestivum*. Cereal Research Communications 5: 365-368

Martín A, Sanchez-Mogue Laguna E (1982) Cytology and morphology of the amphiploid *Hordeum chilense* × *Triticum turgidum* conv. *Durum*. Euphytica 31: 261-267

Martín A, Martín LM, Cabrera A, Ramirez MC, Jimenez MJ, Rubiales D, Hernández P, Ballesteros J (1998) The potential of *Hordeum chilense* in breeding Triticeae species. Triticeae III. Science Publishers, Enfield, New Hampshire, USA. 377-386

Martín A, Cabrera A, Hernánde P, Ramirez MC, Rubiales D, Ballesteros J (2000) Prospect for the use of *Hordeum chilense* in durum wheat breeding Durum wheat improvement in the Mediterranean region: new challenges Vol. 40. Options Méditerranées; Zaragoza Vol. 40)

Martín A, Cabrera A (2005) Cytogenetics of *Hordeum chilense*: current status and considerations with reference to breeding. Cytogenetic and Genome Research 109: 378–384

Martinez-Pérez E, Shaw P, Reader S, Aragón-Alcaide L, Miller TE, Moore G (1999) Homologous chromosome pairing in wheat. Journal of Cell Science 112: 1761-1769

Martinez-Pérez E, Shaw P, Moore G (2001) The *Ph1* locus is needed to ensure specific somatic and meiotic centromere association. Nature 411: 204-207

Martinez-Pérez E, Shaw P, Aragón-Alcaide K, Moore G (2003) Chromosomes form into seven groups in hexaploid and tetraploid wheat as a prelude to meiosis. Plant Journal 33: 21-29

Masterson J (1994) Stomatal size in fossil plants-evidence for polyploid in majority isochromosomes. Genetics 145: 1155-1160

May T, Soll J (2000) 14-3-3 proteins form a guidance complex with chloroplast precursor proteins in plants. Plant Cell 12: 53-64

Mettin D, Bltithner WD, Schlegel R (1973) "Additional evidence on spontaneous 1B/1R wheat-rye substitutions and translocations" (paper presented at the Proc 4th Int Wheat Genet Symp. University of Missouri, Columbia MO, 79-184)

Miller TE, Reader SM, Chapman (1982) The addition of *Hordeum chilense* chromosomes to wheat. In: Proc. Int. Symp. Eucarpia on Induced Variability in Plant Breeding (ed. C Broertjes). Pudoc, Wageningen, p. 79-81

Miller TE (1984) The homoeologous relationship between the chromosomes of rye and wheat. Current status. Canadian Journal of Genetics and Cytology 26: 579-589

Miller TE, Reader SM, Purdie KA, King IP (1994) Determination of the frequency of wheat-rye chromosome pairing in wheat x rye hybrids with and without chromosome 5B. Theoretical and Applied Genetics 89: 255-258

Mironov V, De Veylder L, Van Montagu M, Inzé D (1999) Cyclin-dependent kinases and cell division in higher plants—the nexus. Plant Cell 11: 509–521

Molnár-Láng M, Linc G, Friebe BR, Sutka J (2000) Detection of wheat-barley translocations by genomic *in situ* hybridization in derivatives of hybrids multiplied in vitro. Euphytica 112: 117-123

Mukherjee AB, Cohen MM (1968) DNA synthesis during meiotic prophase in male mice. Nature 219: 489-490

Mullan DJ, Platteter A, Teakle NL, Appels R, Colmer TD, Anderson JM, Francki MG (2005) EST-derived SSR markers from defined regions of the wheat genome to identify *Lophopyrum elongatum* specific loci. Genome 48:811–822

Mullan DJ, Mirzaghaderi G, Walker E, Colmer TD, Francki MG (2009) Development of wheat–*Lophopyrum elongatum* recombinant lines for enhanced sodium 'exclusion'during salinity stress. Theoretical and Applied Genetics 119: 1313–1323

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Research 8: 4321-4326

Nadolska-Orczyk A, Gasparis S, Orczyk W (2009) The determinants of grain texture in cereals. Journal of Applied Genetics 50: 185-197

Neta-Sharir I, Isaacson T, Lurie S, Weiss D (2005) Dual role for tomato heat shock protein 21: protecting photosystem II from oxidative stress and promoting colour changes during fruit maturation. Plant Cell 17: 1829-1838

Niwa O, Simanuki M, Miki F (2000) Telomere-led bouquet formation facilitates homologous chromosome pairing and restricts ectopic interactions in fission yeast meiosis. EMBO Journal 19: 3831-3840

Oettler G (2005) The fortune of a botanical curiosity- Triticale: past, present and future. Journal of Agricultural Science 143: 329-346

Okamoto M (1957) A synaptic effect of chromosome V. Wheat Information Service 5: 6

Ormerod MG, Payne AWR, Watson JV (1987) Improved program for the analysis of DNA histograms. Cytometry 8: 637-641

Owens RA, Diener TO (1981) Sensitive and rapid diagnosis of potato spindle tuber viroid by nucleic acid hybridization. Science 213: 670-672

Page SL, Hawley RS (2004) The genetics and molecular biology of the synaptonemal complex. Annual Review of Cell and Developmental Biology 20: 525-558

Pedersen C, Rasmussen SK, Linde-Laursen I (1996) Genome and chromosome identification in cultivated barley and related species of the Triticeae (Poaceae) by *in situ* hybridization with the GAA-satellite sequence. Genome 39: 93-104

Pedersen C, Langridge P (1997) Identification of the entire chromosome complement of bread wheat by two-color FISH. Genome 40: 589-593

Petronczki M, Siomos MF, Nasmyth K (2003) Un menage a quatre: the molecular biology of chromosome segregation in meiosis. Cell 112: 423-440

Prieto P, Ramirez MC, Ballesteros J, Cabrera A (2001) Identification of intergenomic translocations involving Wheat, *Hordeum vulgare* and *Hordeum chilense* chromosomes by FISH. Hereditas 135: 171-174

Prieto P, Martin A, Cabrera A (2004) Chromosomal distribution of telomeric and telomeric-associated sequences in *Hordeum chilense* by *in situ* hybridization. Hereditas 141: 122-127

Prieto P, Shaw P, Moore G (2004a) Homologous recognition during meiosis is associated with a change in chromatin conformation. Nature Cell Biology 6: 906-908

Qi X, Stam P, Lindhout P (1996) Comparison and integration of four barley genetic maps. Genome 39: 379-394

Qi LL, Friebe B, Zhang P, Gill BS (2007) Homoeologous recombination, chromosome engineering and crop improvement. Chromosome Research 15: 3-19

Qi LL, Pumphrey MO, Friebe B, Chen PD, Gill BS (2008) Molecular cytogenetic characterization of alien introgressions with gene *Fhb3* for resistance to *Fusarium* head blight disease of wheat. Theoretical and Applied Genetics 117: 1155–1166

Rayburn AL, Gill BS (1986) Isolation of a D-genome specific repeated DNA sequence from *Aegilops squarrosa*. Plant Molecular Biology Reporter 4: 102-109

Riehl S, Zeidi M, Conard NJ (2013) Emergence of agriculture in the foothills of the Zagros Mountains of Iran. Science 341: 65-67

Riley R, Chapman V (1958) Genetic control of the cytologically diploid behaviour of hexaploid wheat. Nature 182: 713-715

Riley R, Chapman V (1958a) The production and phenotypes of wheat-rye chromosome addition lines. Heredity 12: 301-315

Riley R (1960) The diploidization of polyploidy wheat. Heredity 15: 407-429

Riley R, Chapman V, Johnson R (1968) Introduction of yellow rust resistance of *Aegilops comosa* into wheat by genetically induced homoeologous recombination. Nature 217: 383-384

Riley R, Chapman V, Johnson R (1968a) The incorporation of alien disease resistance in wheat by genetic interference with the regulation of meiotic chromosome synapsis. Genetics Research 12: 199-219

Rodríguez-Suárez C, Giménez MJ, Atienza SG (2010) Progress and perspectives for carotenoid accumulation in selected Triticeae species. Crop and Pasture Science 61: 743-751

Rodríguez-Suárez C, Giménez MJ, Gutiérrez N, Ávila CM, Machado A, Huttner E, Ramírez MC, Martín AC, Castillo A, Kilian A, Martín, Atienza SG (2012a) Development of wild barley (*Hordeum chilense*)-derived DArT markers and their use into genetic and physical mapping. Theoretical and Applied Genetics 124: 713-722

Rodríguez-Suárez C, Atienza SG (2012b) *Hordeum chilense* genome, a useful tool to investigate the endosperm yellow pigment content in the *Triticeae*. BMC Plant Biology 12: 200

Rodríguez-Suárez C, Mellado-Ortega E, Hornero-Méndez D, Atienza SG (2014) Increase in transcript accumulation of *Psy1* and e-*Lcy* genes in grain

development is associated with differences in seed carotenoid content between durum Wheat and tritordeum. Plant Molecular Biology 84: 659-673

Rubiales D, Ballesteros J, Martín A (1992) Resistance to *Septoria tritici* in *Hordeum chilense* × *Triticum* spp. amphiploids. Plant Breeding 109: 281-286

Rubiales D, Brown JKM, Martín A (1993) *Hordeum chilense* resistance to powdery mildew and its potential use in cereal breeding. Euphytica 67: 215-220

Rubiales D, Martín A (1999) Chromosomal location in *H. chilense* and expression of common bunt resistance in wheat addition lines. Euphytica 109: 157-159

Rubiales D, Reader SM, Martín A (2000) Chromosomal location of resistance to *Septoria tritici* in *Hordeum chilense* determined by the study of chromosomal addition and substitution lines in 'Chinese Spring' wheat. Eyphytica 115: 221-224

Rubiales D, Carver TWL, Martín A (2001) Expression of resistance to *Blumeria graminis* f.sp. *tritici* in " Chinese Spring" wheat addition lines containing chromosomes from *Hordeum vulgare* and *H. chilense*.

Salamini F, Özkan H, Brandolini A, Schäfer-Pregl R, Martín W (2002) Genetics and geography of wild cereal domestication in the near east. Nature Reviews Genetics 3: 429–441

Salic A, Mitchison TJ (2008) A chemical method for fast and sensitive detection of DNA synthesis *in vivo.* Proceedings of the National Academy of Sciences 105: 2415-2420

Salse J, Feuillet C (2007) Comparative genomics of cereals. In Genomics-Assisted Crop Improvement: Volume 1: Genomics Approaches and Platforms (Varshney, R.K. and Tuberosa, R., eds), pp. 177–205, Springer Verlag

Scherrer B, Isidore E, Klein P, Kim JS, Bellec A, Chalhoub B, *et al*., (2005) Large intraspecific haplotype variability at the *Rph7* locus results from rapid and recent divergence in the barley genome. Plant Cell 17: 361-374

Scherthan H (2001) A bouquet makes ends meet. Nature Reviews Molecular Cell Biology 6: 975-987

Schwarzacher T, Leitch AR, Bennett MD, Heslop-Harrison JS (1989) *In situ* localization of parental genomes in a wild hybrid. Annals of Botany 64: 315-324

Sears ER (1956) The transfer of leaf rust resistance from *Aegilops umbellulata*. Brookhaven symposia in biology 9: 1-22

Sears ER, Okamoto M (1958) Intergenomic chromosome relationship in hexaploid wheat, Proc. 10th Int. Cong. Genet., Montreal, Canada. 258-259

Sears ER (1972) Chromosome engineering in wheat. Stadler Genetics Symposium 4: 23-28

Sears ER (1976) Genetic control of chromosome pairing in wheat. Annual Review of Genetics 10:31-51

Sears ER (1977) Induced mutant with homoeologous pairing in common wheat. Canadian Journal of Genetics and Cytology 19: 585-593

Sears ER (1981) Transfer of alien genetic material to wheat in the Wheat Science-today and tomorrow, ed. D. Evans IT and Peacock WI (Cambridge University Press, UK), 75-89

Sears ER (1982) A wheat mutation conditioning an intermediate level of homoeologous chromosome pairing. Canadian Journal of Genetics and Cytology 24: 715-719

Sears ER (1993) Use of radiation to transfer alien chromosome segments to wheat. Crop Science 33: 897-901

Sherman JD, Smith LY, Blake TK, Talbert LE (2001) Identification of barley genome segments introgressed into wheat using PCR markers. Genome 44: 38-44

Sherr CJ, Roberts JM (1999) CDK inhibitors: positive and negative regulators of G1-phase progression. Genes and Development 13: 1501-1512

Shi F, Endo TR (1997) Production of wheat-barley disomic addition lines possessing an Aegilops cylindrical gametocidal chromosome. Genes & Genetic Systems 72: 243-248

Shi F, Endo TR (1999) Genetic induction of structural changes in barley chromosomes added to common wheat by a gametocidal chromosome derived from Aegilops cylindrical. Genes & Genetic Systems 74: 49-54

Shi F, Endo TR (2000) Genetic induction of chromosomal rearrangements in barley chromosome 7H added to common wheat. Chromosoma 109: 358-363

Simchen G (1974) Are mitotic functions required in meiosis?. Genetics 76: 745-753

Smith KN, Penkner A, Ohta K, Klein F, Nicolas A (2001) B-type cyclins CLB5 and CLB6 control the initiation of recombination and synaptonemal complex formation in yeast meiosis. Current Biology 11: 88-97

Stillman B (1996) Comparison of DNA replication in cells from Prokarya and Eukarya. In De Pamphilis, M. L. (ed.) DNA Replication in Eukaryotic Cells. Cold Spring Harbor Laboratory Press, Cold Spring

Strich R (2004) Meiotic DNA replication. Current Topics in Developmental Biology 61: 29-60

Tang Y, Sorrells ME, Kochian LV, Garvin DF (2000) Identification of RFLP markers linked to the barley aluminum tolerance gene Alp. Crop Science 40: 778-782

Tasleem-Tahir A, Nadaud I, Girousse C, Martre P, Marion D, Branlard G (2011) Proteomic analysis of peripheral layers during wheat (*Triticum aestivum* L.) grain development. Proteomics 11: 371-379

Thomson AM, Gillespie PJ, Blow JJ (2010) Replication factory activation can be decoupled from the replication timing program by modulating CDK levels. Cell Biology 188: 209-221

Trelles-Sticken E, Dresser ME, Scherthan H (2000) Meiotic telomere protein Ndj1p is required for meiosis-specific telomere distribution, bouquet formation and efficient homologue pairing. Journal of Cell Biology 151: 95-106

Untergasser A, nijveen H, Rao X, Bisseling T, Geurts R, Leunissen JAM (2007) Pimer3plus, an enhanced web interface to Primer3. Nucleic Acids Research 35: W71-W74

Van Gijlswijk RPM, Zijlmans HJMAA, Weigant J, Bobrow MN, Erickson TJ, Adler KE, Tanke HJ, Raap AK (1997) Fuorochrome-labeled tyramides: use in immunocytochemistry and fluorescence *in situ* hybridization. Journal of Histochemistry and Cytochemistry 45: 375-382

Vassilakos N, Kektsidou OI, Papaeythimiou M, Vervari C (2012) Comparison of direct-RT-PCR and dot-blot hybridization for the detection of Potato spindle tuber viroid in natural host plant species. European Journal of Plant Pathology 134: 85-864

Vrána J, Kubaláková M, Simková H, Cihalikovai J, Lysák MA, Dolezel J (2000) Flow sorting of mitotic chromosomes in common wheat (*Triticum aestivum* L.). Genetics 156: 2033-2041

Wang J, Raman H, Zhou M, Ryan PR, Delhaize E, Hebb DM, Coomes N, Mendham N (2007) High-resolution mapping of the *Alp* locus and identification of a candidate gene HvMATE controlling aluminum tolerance in barley (*Hordeum vulgare* L.) Theoretical and Applied Genetics 115: 265-276

Wang X, Lai J, Liu G, Chen F (2002) Development of a Scar marker for the *Ph1* locus in common wheat and its application. Crop Science 42: 1365-1368

Watanabe Y, Yokobayashi S, Yamamoto M, Nurse P (2001) Pre-meiotic S phase is linked to reductional chromosome segregation and recombination. Nature 409: 359-363

Werner JE, Endo TR, Gill BS (1992) Toward a cytogenetically based physical map of the wheat genome. Proceedings of the National Academy of Sciences 89: 11307-11311

Wicker T, Yahiaoui N, Guyot R, Schlagenhauf E, Liu ZD, Dubcowsky J, Keller B (2003) Rapid genome divergence at orthologous low molecular weight glutenin loci of the A and Am genomes of wheat. Plant Cell 15: 1186-1197

Wicker T, Mayer KFX, Gundlach H, Martis M, Steuernagel B, Scholz U, Simkova H, Kabalakova M, Choulet F, Taudien S. Platzer M, Feuillet C, Fahima T, Budak H, Dolezel J, Keller B, Stein N (2011) Frequent gene movement and pseudogene evolution is common to the large and complex genomes of wheat, barley and their relatives. Plant Cell 23: 1706-1718

Wimber DE, Prensky W (1963) Autoradiography with meiotic chromosomes of the male newt (*Triturus viridescens*) using H^3-Thymidine. Genetics 48: 1731-1738

Xin ZY, Zhang ZY, Chen X, Lin ZS, Ma YZ, Xu HJ, Larkin PJ, Banks PM (2001) Development and characterization of common wheat-*Thinopyrum intermedium* translocation lines with resistance to barley yellow dwarf virus. Euphytica 119: 161–165

Yadav D, Singh NK (2011) Wheat triticin: a potential target for nutritional quality improvement. Asian Journal of Biotechnology 3: 1-21

Yousafzai FK, Al-Kaff N, Moore G (2010) The molecular features of chromosome pairing at meiosis: the polyploidy challenge using wheat as a reference. Functional and Integrative Genomics 10: 147-156

Zarco-Hernández JA, Santiveri F, Michelene A, Peña RJ (2005) Durum wheat (*Triticum turgidum*, L) carrying the 1BL/1RS chromosomal translocation: agronomic performance and quality characteristics under Mediterranean conditions. European Journal of Agronomy 22: 33-43

Zegerman P, Diffley JFX (2007) Phosphorylation of Sld2 and Sld3 by cyclin-dependent kinases promote DNA replication in budding yeast. Nature 445: 281-285

Zeller FJ (1973) 1B/1R substitutions and translocations, Proc 4th Int Wheat Genet Symp. University of Missouri, Columbia MO, 209-221

Zeller FJ, Hsam SLK (1984) Broadening the genetic variability of cultivated wheat by utilizing rye chromatin. P. 161-173. In S. Sakamoto (ed.) Proc. Intl. Wheat Genet. Symp., 6th Kyoto, Japan. 28 Now-3Dec 1983. Plant Germplasm Int., Kyoto University, Kyoto, Japan

Zhang CY, Dong CH, He XY, Zhang LP, Xia XC, He ZH (2011) Allelic variants at the *TaZds-D1* locus on wheat chromosome 2DL and their association with yellow pigment content. Crop Science 51: 1580-1590

Zhao R, Wang H, Xiao J, Bie T, Cheng S, Jia Q, Yuan C, Zhang R, Cao A, Chen P, Wang X (2013) Induction of 4VS chromosome recombinants using the CS *ph1b* mutant and mapping of the wheat yellow mosaic virus resistance gene from *Haynaldia villosa.* Theoretical Applied Genetics 126: 2921-2930

Zickler D, Kleckner N (1999) Meiotic chromosomes: integrating structure and function. Annual Review of Genetics 33: 603-754

Zohary D, Hopf M (2000) Domestication of Plants in the old world, Oxford University Press