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"Introgresión de *Hordeum chilense* en trigo para la mejora del contenido de pigmentos carotenoides en grano"

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TITULO: *Introgresión de Hordeum Chilense en trigo para la mejora del contenido de pigmentos carotenoides en grano*

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TÍTULO DE LA TESIS:

Introgresión de *Hordeum chilense* en trigo para la mejora del contenido de pigmentos carotenoides en grano

DOCTORANDA: Dña. María Gabriela Mattera

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

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

La presente tesis doctoral se ha desarrollado en el marco de los proyectos AGL2011-24399 y AGL2014-53195-R con el objetivo de determinar y utilizar la variabilidad genética de *H. chilense* para la mejora del contenido en carotenoides y su esterificación en grano de trigo.
Durante esta tesis se han desarrollado introgresiones de *H. chilense* en trigo harinero; se han desarrollado estudios genéticos en relación con el efecto de la introgresión de *H. chilense* en trigo sobre el perfil de carotenoides en grano; la localización cromosómica de genes candidato y la diversidad existente en *H. chilense;* y se ha estudiado la formación de ésteres de carotenoides en grano desde un punto de vista genético (evolución durante el desarrollo) como ambiental (influencia de distintas condiciones de temperatura durante el ciclo de cultivo).
La tesis se ha desarrollado de acuerdo con los plazos marcados, lo que ha permitido que hasta el momento ya se hayan publicado 4 trabajos en revistas SCI, y que haya otros dos manuscritos en preparación.

Por todo ello, la tesis cumple los requisitos de calidad necesarios para su exposición y defensa pública.

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

Córdoba, 13 de Febrero de 2017

Firma del/de los director/es

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A los que no pierden la ilusión…

A mi querida familia...

Nota:

A fin de establecer una coherencia formal a lo largo del presente documento, se han editado los trabajos originales y se ha uniformado todo el manuscrito en un único estilo y formato.

Summary

The use of wild relatives in breeding programs of cultivated species provides plant breeders with a pool of useful genes to develop new varieties with better agronomic performance or quality characteristics. Alien resources have been widely used in both bread and durum breeding programs. Endosperm color of wheat grains, which is mainly due to carotenoid accumulation, is an important quality criterion in wheat breeding programs. Tritordeum (the fertile amphiploid between *Hordeum chilense* and durum wheat) shows high yellow pigment content (YPC) in grain due to the contribution of the genes from *H. chilense*, mainly from chromosome $7H^{ch}$. The *phytoene synthase 1* (*Psy1*) gene codes for the enzyme involved in the first step of carotenoid biosynthesis. This gene is located on the short arm of $7H^{ch}$ and it has potential for wheat breeding. However, knowledge about the real potential of *Psy-1* from *H. chilense* in wheat breeding requires the development of introgression lines of this species into wheat background.

Chapter I and IV show the obtention and cytological and molecular characterization of a set of common wheat-*H. chilense* introgression lines involving chromosome $7H^{ch}$. In total, we have developed seven stable lines and another three lines in hemizygous conditions. All the lines were fertile. Different structural rearrangements of chromosome $7H^{ch}$ were described, including centromeric translocations, telosomic addition, deletion and isochromosome. The presence of *Psy1-H^{ch}* gene in those lines carrying short arm of $7H^{ch}$ was confirmed. In addition, the comparative mapping with chromosome 7H from common barley allowed identifying the collinearity break in the distal portion of both arms of $7H^{ch}$ chromosome, including *Psy1* gene.

Chapter II evaluates the possibility that the inversion mentioned above is limited to a specific genotype. We carry out studies of physical mapping of *Psy-1* gene in two *H. chilense* accessions by Single Copy FISH (Fluorescent in situ Hybridization) using direct labeling procedure. Our results suggest that *Psy-1* gene is located on the short arm of $7H^{ch}$ chromosome in both accessions, showing that the collinearity break is not exclusive of one accession.

Chapter III examines the effect of $7H^{ch}$ chromosome or part of it on the YPC in wheat grain. We shows that those lines carrying *Psy-1* gene from *H. chilense* exhibited higher carotenoid and lutein concentration than bread wheat. In addition, we determine that $7H^{ch}$ and 7D chromosomes were involved in lutein esterification and it has suggested that the enzymes codified by these chromosomes have complementary activity. Besides, we corroborate that the long arm of $7H^{ch}$ also contributed to the carotenoid content in grain but only in presence of $7H^{ch}S$.

The incidence of temperature regime during grain development on carotenoid accumulation and lutein esterification profile including fatty acid selectivity (palmitic *vs.* linoleic) and regioselectivity (esterification at positions 3 *vs.* 3') was assessed in chapter V. Our results show that lutein esters are more stable in vivo than free carotenoids and the enzymes codified by $7H^{ch}$ and 7D chromosomes are complementary since they differed in the fatty acid (palmitic and linoleic, respectively) used in the esterification, as well as in the position in which they esterified (regioselectivity 3 and 3', respectively). In addition, we determine that high temperatures promoted the accumulation of lutein esters with linoleic acid as well the synthesis of regioisomers at 3' position.

Chapter VI examines when the lutein esters begin to form. A detailed analysis of pigment accumulation during grain development is shown. This analysis confirms that not lutein esters are produced until the final

stages of grain filling. In addition, we suggest that simultaneous presences of both 7D and $7H^{ch}$ chromosomes results in earlier formation of lutein esters.

Chapter VII shows the characterization for carotenoid composition and lutein profile in a *H. chilense* collection. The results show a wide range of variation for carotenoid content and lutein ester proportions, which indicates this collection is a suitable diversity panel for genetic studies. In addition, we determine that the esterification preference of *H. chilense* towards palmitic acid is not genome specific but genotype-specific.

This thesis delves into the study of the role of genes from *H. chilense* on the carotenoid synthesis and its esters in wheat grain. The introgression lines developed in this thesis constitutes a good donor material for the improving of carotenoid content since the introgression of chromosome $7H^{ch}$ into wheat background increases the carotenoid content. Besides, this thesis has evidence the important role of both 7D and $7H^{ch}$ chromosomes on lutein esters formation and the modulating effect of temperature during grain development on carotenoid content as well as ester profile. Finally, this thesis has advanced in the study of lutein formation during grain development and it has characterized the existent diversity in *H. chilense* for carotenoid profile (and its esters) in grain.

Resumen

El uso de especies silvestres en programas de mejora de trigo ha proporcionado a los mejoradores un conjunto de genes útiles para desarrollar nuevas variedades con características agronómicas o de calidad superiores. El color del endospermo de los granos de trigo está principalmente determinado por la acumulación de pigmentos carotenoides y es un importante criterio de calidad en trigo. El tritórdeo es un anfiploide fértil originado a partir de la cruza entre *Hordeum chilense* y trigo duro y muestra un alto contenido de pigmentos amarillos en grano debido a la contribución de los genes provenientes de *H. chilense*, principalmente del cromosoma $7H^{ch}$. El gen Fitoeno sintasa 1 (*Psy1*) codifica la enzima involucrada en el primer paso de la biosíntesis de carotenoides Este gen se ha sido localizado en el brazo corto del cromosoma $7H^{ch}$ y tiene potencial para la mejora de trigo. Sin embargo, se necesita mayor información para conocer el potencial real del gen *Psy-1* de *H. chilense* en la mejora de trigo y para ello se requiere el desarrollo de líneas de introgresión de esta especie en el fondo genético de trigo.

En los capítulos I y IV se muestra la obtención y la caracterización citogenética y molecular de un grupo de líneas de introgresión de *H. chilense* en trigo harinero, involucrando específicamente al cromosoma $7H^{ch}$. En total, hemos desarrollado siete líneas estables y otras tres líneas en condición de hemicigosis, siendo todas ellas líneas fértiles. Todas las líneas fueron fértiles. Se describen diferentes reordenamientos estructurales del cromosoma $7H^{ch}$, incluyendo translocaciones centroméricas, adiciones telosómicas, una deleción y un isocromoma. Se confirma la presencia del gen *Psy1* en aquellas líneas que llevan el brazo corto del cromosoma $7H^{ch}$. Además, el mapeo comparativo con el cromosoma 7H de cebada ha permitido identificar una inversión en la porción distal de ambos brazos del cromosoma $7H^{ch}$, incluyendo el gen *Psy1*.

En el capítulo II se evalúa si la inversión mencionada anteriormente se limita a un genotipo específico. Se llevan a cabo estudios de mapeo físico del gen *Psy1* en dos accesiones de *H. chilense* mediante el procedimiento de hibridación *in situ* de genes de copia única usando marcaje directo. Los resultados sugieren que el gen *Psy-1* está localizado en el brazo corto del cromosoma $7H^{ch}$ en ambas accesiones, comprobando que la ruptura de la colinealidad no es exclusiva de una accesión.

En el capítulo III se examina el efecto del cromosoma $7H^{ch}$ o parte de él en el contenido de pigmento amarillo del grano de trigo. Se muestra que aquellas líneas que llevan el gen *Psy1* de *H. chilense* exhibieron mayor concentración de carotenoides y luteína que trigo harinero. Además, se determina que los cromosomas $7H^{ch}$ y 7D están involucrados en la esterificación de la luteína y se sugiere que las enzimas codificadas por estos cromosomas tienen actividad complementaria. Asimismo, se comprueba que el brazo largo $7H^{ch}$ también contribuye en el contenido de carotenoides pero sólo en presencia del brazo corto de este cromosoma.

En el capítulo V se evalúa la incidencia de diferentes regímenes de temperatura durante el desarrollo del grano en la acumulación de carotenoides y el perfil de esterificación de la luteína incluyendo la selectividad de ácidos grasos (palmítico o linoleico) y la posición en la que lo esterifican (regioselectividad, posiciones 3 o 3'). Nuestros resultados muestran que los ésteres de luteína son más estables *in vivo* que los carotenoides libres y que las enzimas codificadas por los cromosomas 7D y 7H^{ch} son complementarias ya que difieren tanto en el ácido graso usado en la esterificación (palmítico y linoleico, respectivamente), como en la posición en la que lo esterifican (regioselectividad, posiciones 3 y 3', respectivamente). Además se determina que el incremento de la temperatura favorece la acumulación de los ésteres de luteína con ácido linoleico así como la síntesis de regioisómeros en la posición 3'.

En el capítulo VI se examina cuando los ésteres de luteína comienzan a formarse. Se muestra un análisis detallado de la acumulación de pigmentos durante el desarrollo de grano. Este análisis confirma que los ésteres de luteína no se producen hasta las etapas finales del llenado de grano. Además, se sugiere que la presencia simultánea de ambos 7D y 7H^{ch} resulta en la formación más temprana de ésteres de luteína.

En el capítulo VII se muestra la caracterización de la composición de carotenoides y el perfil de luteína en una colección de *H. chilense.* Los resultados muestran un amplio rango de variación en el contenido de carotenoides y en la proporción de ésteres de luteína, lo cual indica que esta población es un panel de diversidad adecuado para estudios genéticos. Además, se determina que la preferencia de *H. chilens*e hacia la utilización del ácido palmítico en la esterificación de la luteína es específico al genotipo y no al genoma.

Esta tesis profundiza en el estudio del papel de los genes de *H. chilense* en la producción de carotenoides y sus ésteres en grano de trigo. La introgresión del cromosoma 7H^{ch} en fondo genético de trigo incrementa el contenido de carotenoides, de ahí que las líneas de introgresión desarrolladas en esta tesis constituyan un buen material de partida para la mejora de este carácter. Por otro lado, esta tesis ha evidenciado el papel importante de los cromosomas 7D y 7H^{ch} en la formación de ésteres de luteína y el efecto modulador de la temperatura durante el desarrollo del grano tanto en el contenido de carotenoides como en el perfil de ésteres. Por último, esta tesis ha avanzado en el estudio de la formación de ésteres durante el desarrollo del grano y ha caracterizado la diversidad existente en *H. chilense* para el perfil de carotenoides (y sus ésteres) en grano.

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INTRODUCTION

Wheat importance and origin

Wheat is one of the most important cereals in the world, with a production around 728 million tons. It is the third most-produced cereal behind corn and rice (FAO, 2014). China is the major wheat producer reaching 17.3% of world production, followed by India (13.0%), Russian Federation (8.2%), United States of America (7.6%) and France (5.4%). Despite wheat is a dominant crop in temperate areas, it exhibits a huge adaptability across different environments and it represents the most widely grown food, reaching a global harvested area of almost 222 MHa in 2014 (FAO). Spain ranked as the twenty-second wheat producer in 2014, with a production level of 6,471,400 tons from a total harvested area of 2,171,200 hectares, down three positions regarding the production in 2013, when yields per hectare were 20% higher. Wheat accounts for 31.5% of total cereal production in Spain. Wheat production includes bread wheat, which represents 80.9% of the total production, and durum wheat, with the remaining 19.1% (Eurostat data). Wheat production in Argentina reached 13,930,078 tons in 2014 corresponding to a total harvested area of 4,957,300 hectares. With this production, Argentina was ranked as the fourteenth wheat producing country in the world, rising four positions compared to 2013.

Wheat is considered a staple food, together with rice and corn. Around 50% of the calories consumed by humanity come from these three cereals: rice (23%), wheat (17%) and corn (10%) (Khush 2003). Products derived from wheat grain are an important source of carbohydrates, protein and fiber. In addition, they provide a low amount of lipids (saturated, mono-unsaturated and poly-unsaturated) and a wide range of minerals (calcium, iron, magnesium, phosphorus, potassium, sodium and zinc) and vitamins (thiamine, riboflavin, niacin, vitamin B-6, folate, vitamin E-alpha-tocopherol and vitamin K-phylloquinone) as reported by United States Department of Agriculture Food Composition Databases (https://ndb.nal.usda.gov/ndb/).

Wheat grain was utilized since ancient times. Archaeological evidence of domesticated cereals remains is dated more than 9,000 years BC (Nesbitt and Samuel 1995). The first document registering the breadmaking process from wheat grain dates to 2,600 B.C. Egyptians were the first to utilize yeast for fermentation and expansion of dough in breadmaking (Harlan 1981). Later, romans established the production at industrial level in the Mediterranean basin. The Industrial Revolution allowed the mechanization of breadmaking, which enabled the development of new products and processes. In this context, new varieties adapted to mechanical processing and high yield were demanded. During the twentieth century, wheat was subjected to intensive breeding programs to develop highly productive varieties, adapted to a wide range of environments and farming systems and with a good baking and semolina quality.

At present, wheat is used to produce a wide number of products including bread, cookies, biscuits, breakfast cereals, pasta, noodles, couscous and beer, among others. Furthermore, wheat is utilized in animal feeding, in cosmetic and pharmaceutical industries and as raw material to produce bioethanol.

Triticum aestivum spp. aestivum L. em. Thell. and *Triticum turgidum spp. durum* Desf. em. Husn are the most cultivated wheat species worldwide and they are known as bread and durum wheat, respectively. Besides, other wheat species are cultivated in restricted areas, including einkorn, emmer, spelt and club, among others. Speciation of *Triticum spp.- Aegilops spp.* complex (*Poaceae* family, *Pooideae* subfamily, Triticeae tribe) is a clear example of allopolyploidization process that took place along its evolution. This complex includes wild and cultivated species, with a chromosome basic number of 7 and three different levels of ploidy: diploid: including one genome and fourteen chromosomes (2n = 2x = 14); tetraploid: including two genomes and twenty-eight chromosomes (2n = 4x = 28); and hexaploid: including three different genomes and forty-two chromosomes (2n = 6x = 42).

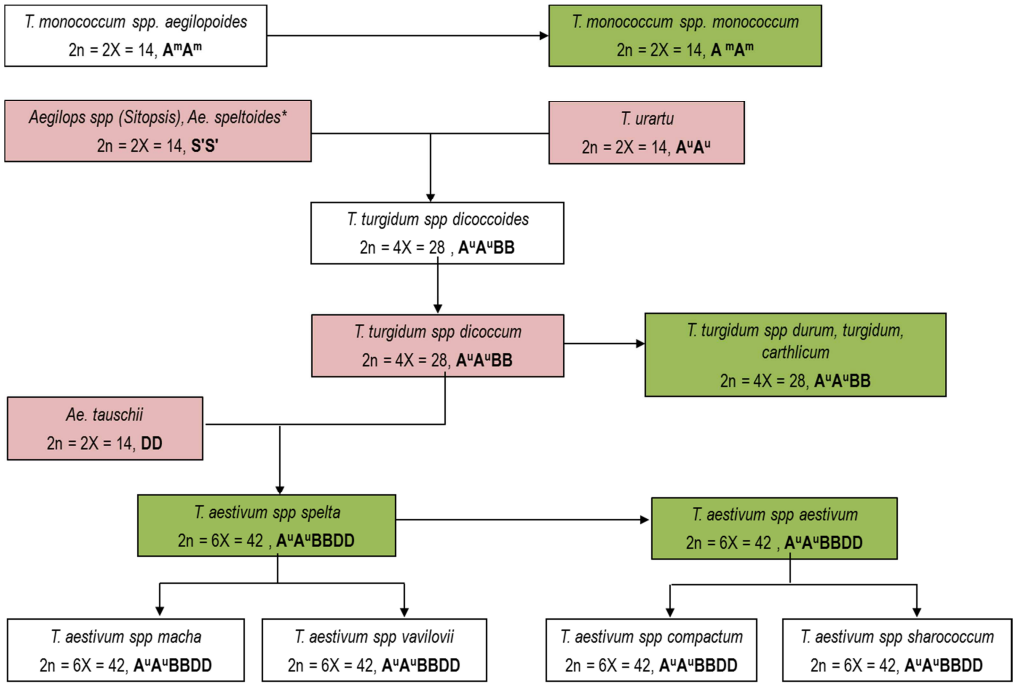

Figure 1. General scheme of the polyploid origin of wheat based on Dvorak *et al.* (2012) and Matsuoka (2011). Species framed in purple are donators of wheat genomes. Species framed in green has been cultivated.

Wheat was originated by a polyploidization process which has been studied and discussed by several authors (Figure 1) (Dvorak *et al.* 2012; Matsuoka 2011; McFadden and Sears 1946). Until now, *T. monococcum* L *ssp. monococcum* (**A^mA^m**) (einkorn) is the only diploid species that has been cultivated and it derives from the domestication of *T. monococcum spp. aegilopoides* Link em. Thell. (syn. *T. boeticum* Boiss.) (Faris 2014). The most accepted hypothesis suggests that *T. urartu* Thum. ex Gandil (**A^uA^u**), is the origin of the **A** genome in polyploid wheat (Baum and Grant Bailey 2004; Dvorak *et al.* 1993). The cross between *T. urartu* and *Aegilops spp* species originated the wild emmer (*T. turgidum spp. dicoccoides* Körn. Ex Asch & Graebner em. Thell.; 2n = 4X = 28, **A^uA^uBB**) which then gave rise to cultivated emmer (*T. turgidum spp. dicoccum* Schrank em Thell.). Several studies have proposed *Ae. speltoides* Tausch. (2n = 2X = 14, SS) as the probable donor of **B** genome and the cytoplasm of most tetra- and hexaploid species (Dvorak *et al.* 2012; Dvorak and Zhang 1990, 1992). Later, durum wheat was originated from cultivated emmer (Matsuoka 2011). Bread wheat was domesticated about 10,000 years

ago (Dubcovsky and Dvorak 2007). It arose by spontaneous hybridization of cultivated tetraploid wheat (A^uA^uBB) with the wild species *Ae. tauschii* Coss. (2n = 2x = 14, **DD**) in the Caspian Sea area (Dvorak *et al.* 1998; McFadden and Sears 1946; Wang *et al.* 2013).

Interspecific hybridization in wheat breeding

Wheat breeding has focused on the development of high yielding varieties characterized by uniformity and adaptation to intensive farming conditions (Esquinas-Alcazar 2005). Local and traditional varieties have been relegated to marginal areas while the intensive selection process led to a marked reduction in the genetic diversity of modern cultivars (Negri *et al.* 2009; Reif *et al.* 2005). It constitutes a serious vulnerability against sudden changes in environmental conditions and the emergence of new pests and diseases (Esquinas-Alcázar 2005). The continued loss of variability has prompted the adoption of several strategies to preserve the available genetic diversity. Furthermore, the enrichment of crop genetic bases is essential for present and future needs (Borlaug 2007; Reif *et al.* 2005). In addition to this, the wide range of wheat-derived products produced at present offers new possibilities for the development of wheat varieties with different quality attributes.

The success in breeding is dependent on the existence of enough genetic diversity for the trait(s) of interest. Wild relatives can be used for wheat breeding but genetic compatibility represents one of the most important limits for plant breeders. Harlan and deWet (1971) established a system to classify the genetic diversity available to breeders based on the compatibility and crossability among genotypes. The genetic diversity available in the primary gene pool is the most accessible since it includes individuals of the same biological species, thus gene transfer is generally simple. The secondary gene pool includes all biological species that will cross with the crop and thus gene transfer is possible. However, the utilization of this genetic diversity is more difficult since it requires overcoming reproductive barriers. Thus, hybrids tend to be sterile and some of them may be weak and difficult to bring to maturity. Finally, a tertiary gene pool is defined, where the crosses with the crop are possible but the resulting hybrids are generally anomalous, lethal, or completely sterile. Thus, in these two last cases, different approaches have been used to get a successful genetic transference.

In introgression programs of wild traits into the cultivated species, the aim is to produce plants with the introgressed trait in the background of the cultivated species. This is usually done by backcrossing the hybrids carrying the favourable trait with the cultivated species. The first step to successful transfer alien chromatin to wheat is the obtention of an interspecific hybrid between the alien species used as a donor and the wheat cultivar. Embryo rescue had an important role in the generation of interspecific hybrids. Synthetic amphiploid lines have been also developed by doubling the chromosome number of sterile F1 hybrid plants. These fertile amphiploid lines derived from hybrids between wheat and their wild relatives have been used as genetic stocks in breeding programs, and as bridging materials for the transfer of

desirable traits from wild species into cultivated ones. In addition, the fertile amphiploid lines have led to novel crops, such as triticale, a new crop derived from rye (*Secale cereale* L.) and wheat.

Alien chromosome addition and substitution lines have been obtained by backcrossing hybrid or amphiploids with wheat. These lines have been widely used in breeding programs. The advantages of using these genetic stocks involve the possibility of assigning species-specific genes or characteristics to particular chromosomes and the potential to transfer desirable agronomic traits among species.

Several researchers have developed specific techniques with the aim of overcome the obstacles that affect the ability to achieve the successful pairing and recombination among genomes of related species, such as the use of irradiation, *Ph1* (pairing homologous) gene and gametocidal genes. In 1956 Ernest Sears succeeded in introducing a small fragment from *Ae. umbellulata* on wheat chromosome 6B carrying a leaf rust resistance gene using X-rays treatment (Sears 1956). This work was pioneer in chromosome manipulation of wild genetic resources for wheat breeding. *Ph1* locus is the main responsible of homologous pairing and recombination during meiosis and it is located on the long arm of 5B chromosome in wheat. Homoeologous paring is induced in the absence of *Ph1* gene and the recombination among distant species could occur. *Ph1* mutants, *ph1bph1b* and *ph1cph1c,* were identified in hexaploid (Sears 1977) and tetraploid (Giorgi 1978) wheat, respectively. The *Ph1* mutants have been used to induce homoeologous chromosome pairing among Triticeae species and they constitute a method of genes transferring from relative species to wheat.

Endo (1988) has made a significant contribution in the obtention of alien-wheat stocks through development of 'gametocide system' as the way to generate structural changes in both wheat and their wild relatives. This system is based on the introgression of 'gametocide' chromosomes (Gc) into bread wheat, which came from species of genus *Aegilops* and they can induce semi-lethal chromosome breaks in gametes not containing the gametocidal chromosome (Figure 2). Many cytogenetic stocks carrying structural changes on alien chromosomes (including translocation and deletion) into wheat genetic background were obtained by this method.

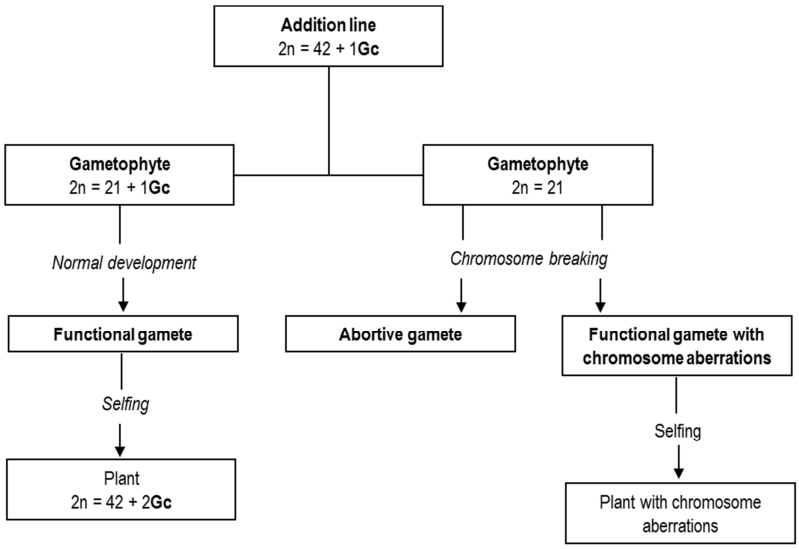

Figure 2. Scheme based on Endo *et al.* (2011) representing the action of gametocidal chromosome added to bread wheat genetic background.

The potential of wild relatives for the improvement of resistance and tolerance to biotic or abiotic stress, as well as enhancement of quality traits, has been evidenced in both common wheat (Khlestkina 2014) and durum (Ceoloni *et al.* 2014). An example of alien introgression in wheat involves the wheat-rye (1RS/1BL) translocation, which confers resistance to many diseases and is widely used in wheat breeding programs.

In addition, alien-wheat cytogenetic stocks have been very useful in genetic studies including physical mapping of gene(s) or molecular makers and the development of deletion mapping (Ceoloni *et al.* 2014; Khlestkina 2014; Molnár-Láng *et al.* 2014). Thus, the development of this type of genetic resources is an interesting tool for researchers and breeders.

Potential of Hordeum chilense Roem. et Schultz. for wheat improvement

The development of crosses between wheat and barley (*Hordeum vulgare* L.) has been a major goal for wheat breeders since it would allow the incorporation of favorable traits from barley to wheat. Although the continuous effort to transfers useful genes from barley to wheat has resulted in the development of wheat-barley translocation lines (reviewed by Molnár-Láng *et al.* 2014), the difficulties in obtaining wheat-*H. vulgare* amphiploids promoted the utilization of other species of the genus *Hordeum,* such as *H. chilense*.

H. chilense Roem et Schultz (2n=2x=14, $H^{ch}H^{ch}$) (Figure 3) is a wild species distributed from 29º to 43º Latitude South and from sea level to 1,800 m of altitude (Martín *et al.* 1996). It is a weak perennial, included in natural pastures and very appreciated by cattle (Valderrama *et al.* 1991). A number of agronomically interesting characteristics have identified in *H. chilense*, such as biotic and abiotic stress resistance (Martín *et al.* 1999), variability for seed storage proteins (Álvarez *et al.* 2001; Atienza *et al.* 2002; Atienza *et al.* 2000), high carotenoid content (Álvarez *et al.* 1998; Rodríguez-Suárez *et al.* 2010) and cytoplasmic male sterility (Martín *et al.* 2008).

Indeed, hexaploid and octoploid tritordeum were obtained from crosses between *H. chilense* and both durum and bread wheat, respectively (Martín and Chapman 1977; Martín and Sánchez-Monge 1982). Hexaploid tritordeum exhibits interesting agronomic characteristics (Martín *et al.* 1996) which had promoted the development of a breeding program to use tritordeum as a new crop.

Figure 3. Vegetative and reproductive spikes and seeds of *H. chilense*.

The development of wheat-*H. chilense* chromosome addition and substitution lines (Miller *et al.* 1982) has been very useful for genetic studies in *H. chilense* and they have also allowed the location of specific genes responsible of several agronomic traits on specific *H. chilense* chromosomes. They include those for prolamine content (Payne *et al.* 1987; Tercero *et al.* 1991) and resistance *Schizaphis graminum* (Castro *et al.* 1996) on chromosome $1H^{ch}$; salt tolerance on $1H^{ch}$, $4H^{ch}$ y $5H^{ch}$ (Forster *et al.* 1990); resistance to *Septoria tritici* on $4H^{ch}$ (Rubiales *et al.* 2000); carotenoid content on $2H^{ch}$ y $7H^{ch}$ (Álvarez et al. 1998; Atienza et al. 2004; Rodríguez-Suárez *et al.* 2012) and fertility restoration on $6H^{ch}$ (Martín *et al.* 2008). Wheat- *H. chilense* introgressions lines have also been used to transfer alien chromosome segments carrying genes of interest such as the resistance to cereal root knot nematode (*Meloidogyne naasi*) from *H. chilense* to wheat (Person-Dedryver *et al.* 1990) and potential resistance to *Septoria tritici* blotch from $4H^{ch}$ to durum wheat (Calderón *et al.* 2012).

The first genetic maps of *H. chilense* was developed using a F_2 mapping population derived from the cross between two different accessions, H1 and H7 (Hernández *et al.* 2001). These maps were used to perform the first QTL (Quantitative Trait Loci) analyses in this species (Atienza *et al.* 2004; Vaz Patto *et al.* 2003) and they were constructed with AFLP (Amplified fragment length polymorphism), RAPDs (Random Amplification of Polymorphic DNA) and RFLP (Restriction fragment length polymorphism). Similarly, AFLP markers were also used to study rust resistance in the *H. chilense* germplasm collection (Vaz Patto *et al.* 2001). More recently, a new genetic map has been contructed using the RIL population (H1×H7) and DArT (Diversity Arrays Technology) markers (Rodríguez-Suárez *et al.* 2012). In this case, the map covered 1.503,5 cM and included nine linkage groups assigned to all *H. chilense* chromosomes, greatly improving the genomic coverage of previous maps. Nevertheless, two small linkage groups from $1H^{ch}$ y $7H^{ch}$ chromosomes could not be linked to the rest (Rodríguez-Suárez *et al.* 2012). This DArT-based map allowed the localization of carotenoid-related genes in *H. chilense* (Rodríguez-Suárez and Atienza 2012).

Chromosome addition lines of *H. chilense* in common wheat may serve as tools for the transfer of wild barley genes to wheat. Also, chromosomes from *H. chilense* could be introduced into common wheat

through wheat-tritordeum hybrids and backcrossing to wheat (Martín *et al.* 1998). Introgressed segments can be assessed by *in situ* hybridization, which readily distinguishes *H. chilense* chromosomes from those of wheat (Cabrera *et al.* 1995; González and Cabrera, 1999). The construction of physical map of *H. chilense* containing molecular markers capable of detecting small fragment of chromatin from this species in a wheat background would also enhance the use of this wild species to increase wheat genetic resources. Specific molecular markers have been developed for *H. chilense* chromosome $1H^{ch}$ (Cherif-Mouaki et al, 2011), $3H^{ch}$ (Said *et al.* 2012) and $4H^{ch}$ (Said and Cabrera, 2009) which would be very useful for marker-assisted introgression of these *H. chilense* chromosomes into wheat background.

Grain colour and carotenoid compounds

One of the main attributes of *H. chilense* is its high seed carotenoid content which confers a desirable yellow colour to tritordeum flour (Atienza *et al.* 2007a). Grain colour is an important quality criterion in both durum and common wheat. The main use of durum wheat is focused on pasta production due to high protein content and vitreousness directly related with hardness and compactness (Ficco *et al.* 2014; Troccoli *et al.* 2000). In addition, an intense yellow-amber of semolina is a quality prerequisite to pasta making (Borrelli *et al.* 2003) and it is highly associated with pasta consumer choices (Dexter and Marchylo 2001). There are also specific demands of bread elaborated from durum wheat grains, traditionally from south regions of Italy where currently exits products with appreciation at the level of European market, such as 'Pane di Altamura', 'Pagnotta del Dittaino' and 'Pane di Matera'. Regarding bread wheat derived products, there is certain variability on colour requirements of flour although white flours are usually preferred for breadmaking. However, intensive yellow flour is beneficial for yellow-alkaline noodles production. Besides, from bright white to creamy white colours are required in the production of white noodles salty (Mares and Campbell 2001).

Yellow colour of wheat endosperm directly depends on carotenoid content (Ficco *et al.* 2014; Rodríguez-Suárez *et al.* 2010) but oxidative degradation by lipoxygenase (LOX) and polyphenol oxidase (PPO) enzyme activities also affect this trait (Borrelli *et al.* 1999; Feillet *et al.* 2000)

Carotenoids are C40 isoprenoids with lipophilic nature and they constitute one of the most important organic pigments (Hirschberg 2001). These compounds are only produced by photosynthetic organisms and some non-photosynthetic bacteria, yeast and molds; whereas the animals can only obtain them by eating plant foods (Nisar *et al.* 2015). They are classified in two groups according to its structure: carotenes, simple hydrocarbons, and xanthophylls, which have oxygen atoms in their terminal rings (hydroxy, epoxy and keto) (reviewed by Fraser and Bramley 2004; Giuliano *et al.* 2008). Carotenoids play basic biological functions as light collectors in the photosynthetic apparatus and protectors against excessive light and oxidative damage (Choudhury and Behera 2001; Demmig-Adams and Adams 2006). In addition, several studies confirmed the benefits of the carotenoid pigments related to human health and

their antioxidant activity (reviewed by Rao and Rao 2007)). Despite cereals have significantly lower carotenoid contents than vegetables and fruits, they have an important impact in the nutritional status of consumers, especially in developing countries, since they contribute the 50% of daily calories (Graham and Rosser 2000). This has promoted the development of biofortification programs in rice (http://www.goldenrice.org/) and maize (http://www.harvestplus.org/) (http://www.afru.udl.cat/). Carotenoids have been implicated in inhibition of carcinogenic processes, increase of immune responses and cellular defense against reactive oxygen species (ROS) and free radicals, and risk reduction of development of cardiovascular and other degenerative diseases (Al-Delaimy *et al.* 2005; Fraser and Bramley 2004). Furthermore, carotenoid compounds carrying a β-ring (β-carotene, zeaxanthin y β-cryptoxanthin, among others) are also vitamin-A precursors (reviewed by Fraser and Bramley 2004). Regarding human health, vitamin A deficiency (VAD) is one of the major causes of malnutrition in the world. As many as 500 000 children become blind due to VAD each year with many of them dying from VAD-related illness within 1 year (data from the World Health Organization; www.who.int/nutrition/topics/vad/en/, accessed November 2016). In addition, an inverse correlation between the progression of degradation of the eye macula and cataract prevalence and the consumption of foods rich in lutein and zeaxanthin has been well documented (Carpentier *et al.* 2009; Olmedilla *et al.* 2001).

Grain colour is an important quality criterion in wheat breeding programs. Three methodologies are predominantly used to determine this trait (reviewed by Rodríguez-Suárez *et al.* 2010): (**a**) chemical extraction of pigments by solvents (n-butanol) and later spectrophotometry determinations (AACC 14-50 Method, AACC2000); (**b**) automatically measurements by reflectance colorimeter (CIE, color space system L*, a* and b*, with b* values providing a measure of yellowness); (**c**) carotenoid determination by High Performance Chromatography (HPLC). Both first methods give colour measurements but they are an indirect measure of the carotenoid content. However, HPLC is more powerful since it allows the determination of the specific carotenoids which is desirable from a nutritional point of view and it shows very good correlation with grain colour (Digesù *et al.* 2009; Fratianni *et al.* 2012).

Biosynthesis of carotenoid compounds

Carotenoid biosynthesis pathway has been detailed by several authors (Giuliano *et al.* 2008; Hirschberg 2001; Matthews and Wurtzel 2007; Nisar *et al.* 2015) (Figure 4). Geranylgenaryl diphosphate (GGPP) is the immediate precursor of carotenoid pigments. Two GGPP (C_{20}) molecules are condensed by *phytoene synthase* (PSY) to produce phytoene (C_{40}), as 15-*cis* isomer. Then, four double bonds are introduced into phytoene (without colour) through four desaturation reactions catalyzed by *phytoene desaturase* (PDS) and ς*-carotene desaturase* (ZDS), to produce lycopene (red colour). These reactions originate poly-*cis* products, which are then converted in *trans* forms by means of two isomerase enzymes, *15-cis-zetacarotene isomerase* (ZISO) and carotene isomerase. From here carotenoid metabolism is

divided in two branches and two different products are generated, depending on the position of double bounds in the hexane ring. On the one hand, lycopene is converted into γ-carotene by *lycopene β-cyclase* enzyme (LCY-B) and that is subsequently converted in β-carotene. On the other hand, *lycopene ε-cyclase* enzyme (LCY-E) catalyzes the reaction to generate δ-carotene from lycopene, which then originates α-carotene through the action of LCY-B. From here, lutein and zeaxanthin are originated by hydroxylation (HYD, hydroxylases) of α- y β-Carotenes, respectively. Then, violaxanthin is derived from zeaxanthin through a reversible epoxidation reaction (ZEP, zeaxanthin epoxidase; VDE, violaxanteno de-epoxidase); which produces anteroxanthin as intermediate product. Neoxanthin is sintetized from violaxanthin by neoxanthin synthase enzyme (NXS).

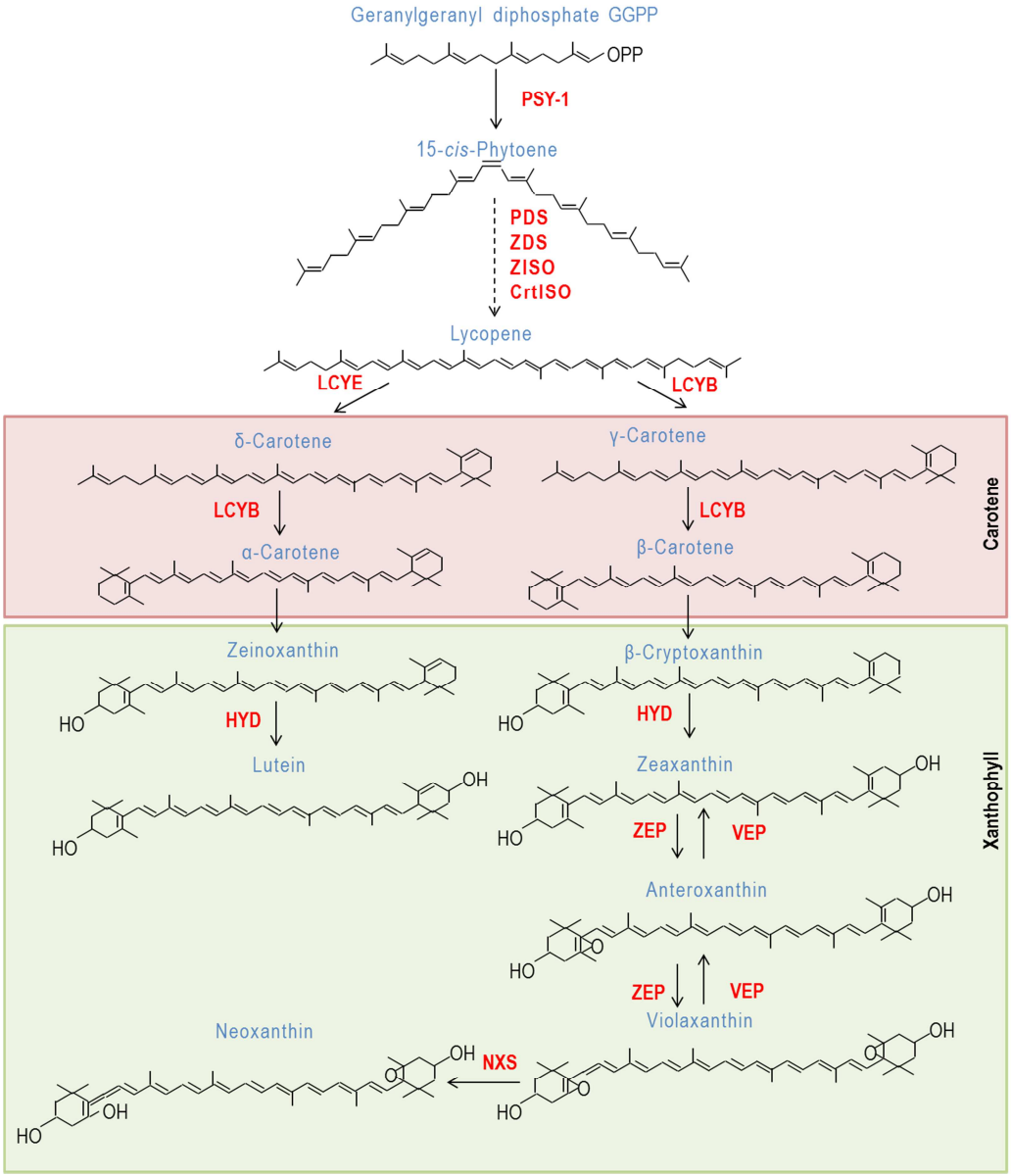

Figure 4. Simplified schematic of the biosynthetic pathway of carotenes and xanthophylls based on Matthews and Wurtzel (2007) and Giuliano *et al.* (2008). Enzymatic reactions are indicated with arrows and the dashed lines indicate the participation of several enzymes. Enzymes involved: PSY-1, phytoene synthase 1; PDS, phytoene

desaturase; ZISO, 15-cis-zetacaroteno isomerase; CrtISO, carotene isomerase; LCYB, lycopene cyclase β; LCYE, lycopene ε-cyclase; HYD, which include carotene hydroxylase β and ε rings; ZEP, zeaxanthin epoxidase; VDE, violaxanteno de-epoxidase; NXS, neoxanthin synthase.

Genetics of yellow pigment content in wheat

Yellow pigment content (YPC) is a quantitative trait. Although it is influenced by the environment it shows a high heritability, between 0.78 and 0.96 in *Triticum* species (Blanco *et al.* 2011; Ziegler *et al.* 2015). Several studies identified QTLs on different chromosomes of durum and common wheat (Blanco *et al.* 2011; Mares and Campbell 2001; Patil *et al.* 2008). However, the loci located on homoeologous group 7 chromosomes are the main responsible of this trait (Atienza *et al.* 2007a; Elouafi *et al.* 2001; Parker *et al.* 1998; Patil *et al.* 2008; Zhang *et al.* 2008). Furthermore, *Phytoene syntase 1* (*Psy-1*) gene was considered as the candidate gene to explain YPC variations in wheat grain, since it was located on chromosomes 7A and 7B (Atienza *et al.* 2007a). Indeed, it co-localized with a QTL for YPC (Pozniak *et al.* 2007) and later studies showed that allelic variants of *Psy-A1* and *Psy-B1* are associated with YPC (He *et al*. 2009; Singh *et al.* 2009; Zhang and Dubcovsky 2008). In addition, co-dominant functional markers were developed for each allelic variant of *Psy-1* gene, which is helpful in breeding programs to increase carotenoid content in durum wheat (He *et al.* 2009; Singh *et al.* 2009) and common wheat (He *et al.* 2008; Howitt *et al.* 2009). *Psy-1* gene has two paralogs, *Psy-2* and *Psy-3* (Li *et al.* 2008; Wurtzel 2004), and all of them share the same structure of six exons and five introns (Wang *et al.* 2009) and they exhibit tissue specific expression. However, only *Psy-1* is related with carotenoid contents in endosperm (Li *et al*. 2009) which holds true in *H. chilense. Psy-1* gene is located on short arm of $7H^{ch}$ chromosome, whereas *Psy-2* and *Psy-3* were located on $5H^{ch}$ (Atienza *et al.* 2007a; Rodríguez-Suárez *et al.* 2012). Furthermore, transcriptomic analyses showed that *Psy-1* and lycopene ε-cyclase (*ε-Lcy)* genes are associated with the superior carotenoid contents of tritordeum grains compared to durum wheat, which shows the potential use of those genes in carotenoid enhancement of wheat grains (Rodríguez-Suárez *et al.* 2014). The results obtained so far in tritordeum suggest that *H. chilense* genes, in particular *Psy-1*, have a high potential for the enhancement of carotenoid content in wheat. However, the real potential of this gene has to be evaluated in euploid combinations, since its introgression would likely result in the elimination of a homoeologous gene.

Carotenoid content in wheat and the importance of H. chilense

Modern durum wheat breeding programs have focused on the development of new varieties with high yellow pigment content (YPC) (Digesù *et al.* 2009; Ficco *et al.* 2014; Troccoli *et al.* 2000) and low oxidative enzyme (LOX and PPO) contents (Borrelli *et al.* 1999; Leenhardt *et al.* 2006). This change was driven by the requirements of the pasta industry, but it is also interesting to improve the nutritional and functional properties of wheat derived products (Digesù *et al.* 2009) since carotenoids consumption is associated with health.

Carotenoid profile in wheat grains, as in other cereals, is mainly constituted lutein representing between 86 to 94% of total carotenoids (Abdel-Aal 2007; Zeigler et al 2015). Minor concentrations of other carotenoids such as zeaxanthin, **β**-criptoxanthin, **α** and **β** carotenes have also been reported (Abdel-Aal 2007; Digesù *et al.* 2009; Fratianni *et al.* 2012; Panfili *et al.* 2004). Lutein is found in all grain fractions, whereas both carotenes (**α** y **β**) and zeaxanthin are only synthesized in the germ and the bran (**α** y **β**) (Ndolo and Beta 2013).

Lutein content is highly diverse among *Triticum* spp. species (Ziegler *et al.* 2015). Lutein is usually reported in its free form although lutein esters have been found in tritordeum (Atienza *et al.* 2007b; Mellado-Ortega and Hornero-Méndez 2012) and common wheat (Ahmad *et al.* 2013; Ziegler *et al.* 2015). Einkorn grains exhibited the highest lutein content in comparison with the other species of *Triticum*, with values 2- 5 higher than those obtained by durum and common wheat grains (Abdel-Aal 2007; Hidalgo *et al.* 2006). In durum wheat varieties, lutein concentration is significantly higher than bread wheat (Abdel-Aal 2007; Ziegler *et al.* 2015). In addition, modern varieties of durum wheat exhibited higher lutein content than wild tetraploids from which they were derived since the former were selected for high YPC (Digesù *et al.* 2009). On contrary, common wheat varieties show the lowest lutein content among *Triticum* species since they have been traditionally selected to provide white flours (Abdel-Aal 2007; Hidalgo *et al.* 2006).

Hexaploid tritordeum exhibited more than 8 times the carotenoid content found in their durum wheat parents (Atienza *et al.* 2007b) due to the contribution of *H. chilense* genome (Álvarez *et al.* 1999). In particular, the addition of the chromosome arm 7H^{ch}S to common wheat significantly increased YPC (Álvarez *et al.* 1998). Along with their high carotenoid contents, tritordeum grains also show a distinctive lutein esterification profile, including mono- and diesters; whereas only small amounts of lutein monoesters were found in durum wheat (Atienza *et al.* 2007b). Mellado-Ortega and Hornero-Mendez (2015a) have shown that *H. chilense* genome is responsible for the characteristic pattern of lutein esterification. Two fatty acids, linoleic and palmitic acids, were involved in lutein esterification. Both lutein monoesters and diesters, including their regioisomers, were identified (Mellado-Ortega and Hornero-Méndez 2015). The presence of lutein esters in tritordeum is important since esterification ability may play a role in carotenoid accumulation. Indeed, esterification is a common means to sequester carotenoids in flower and plants, since esterification with fatty acids increases lipophilic properties and integration into lipid-rich plastoglobules (Hornero-Mendez and Minguez-Mosquera 2000; Vishnevetsky *et al.* 1999a; Vishnevetsky *et al.* 1999b). Few studies have addressed the synthesis of lutein esters in wheat and related cereals and they are mainly limited to changes during seed storage (Kaneko *et al.* 1995; Kaneko and Oyanagi 1995). Although previous studies have shown strong evidences for xanthophyll acyltransferase enzymes in *H. chilense* (Mellado-Ortega and Hornero-Méndez 2012), the possibility of lutein esters being directly proportional of total lutein content remains an open question.

In addition, an important positive effect of temperature on the esterification of lutein during flours and grains storage has been shown (Ahmad *et al.* 2013; Mellado-Ortega *et al.* 2015; Mellado-Ortega and Hornero-Méndez 2016a, b). Considering the high temperatures during grain development in

Mediterranean environments, it is worth investigating whether higher temperatures during grain filling may have a positive impact on lutein ester content. Besides, information on when lutein esters begin to synthesize in tritordeum grains would be useful for transcriptomic and genetic studies but so far this information is not available although no lutein esters prior to 25 days post anthesis have been detected in previous works (Rodríguez-Suárez *et al.* 2014).

As described above, esterification may be important for lutein accumulation. In addition to this, lutein esters are more stable than free lutein (Subagio *et al.* 1999). Similar findings have been reported in cereal grains during post-harvest storage (Ahmad *et al.* 2013; Mellado-Ortega *et al.* 2015). Losses of lutein can be high during storage particularly at high temperatures (Hidalgo and Brandolini 2008; Hidalgo *et al.* 2010), and thus the protective role of lutein esters is important. Accordingly, esterification may be useful to improve lutein retention through the food change since it increases lutein stability at high temperatures. Thus, a better understanding of the genetic control of lutein esterification would be useful to improve lutein retention in cereal-derived products. In this context, the germplasm collection of *H. chilense* may be an important resource since the esterification ability of tritordeums is derived from *H. chilense* genome (Mellado-Ortega and Hornero-Méndez 2015). Indeed, a subset of this collection was analyzed for yellow pigment content (Álvarez *et al.* 1999) and it showed a significant range of variation. Accordingly, the complete *H. chilense* collection may constitute an excellent diversity panel for carotenoid content and it will likely differ for the esterification profile.

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OBJETIVES

OBJETICVES AND HYPOTHESIS

The hypothesis of this thesis is:

Genes related to carotenoids content and located on chromosome 7 H^{ch} from *H. chilense* may serve to improve content of these pigments in wheat grain.

Objectives:

1. To develop introgression lines of *H. chilense* chromosome $7H^{ch}$ into wheat genetic background and their characterization using fluorescence *in situ* hybridization and molecular markers (Chapter I corresponding to published article: 'Cytological and molecular characterization of wheat-*Hordeum chilense* chromosome $7H^{ch}$ introgression lines', Mattera *et al.* 2015; and Chapter IV corresponding to published article: 'Characterization of a set of common wheat-*Hordeum chilense* chromosome $7H^{ch}$ introgression lines and its potential use in research on grain quality traits', Mattera MG and Cabrera A. 2017).
2. To evaluate the effect of chromosomes $5H^{ch}$ and $7H^{ch}$ in common wheat background on total carotenoid content and lutein esterification profile. Additionally, the role of the wheat homoeologous chromosome substituted will be also investigated (Chapter III corresponding to published article: 'Lutein esterification in wheat endosperm is controlled by the homoeologous group 7, and is increased by the simultaneous presence of chromosomes 7D and $7H^{ch}$ from *Hordeum chilense'*, Mattera *et al.* 2015).
3. To assess the modulating effect of different temperature during grain filling on carotenoid content and lutein esterification (Chapter V corresponding to published article: 'Lutein ester profile in wheat and tritordeum can be modulated by temperature: Evidences for regioselectivity and fatty acid preferential of enzymes encoded by genes on chromosomes 7D and $7H^{ch}$', Mattera *et al.* 2017).
4. To investigate when lutein esters initiate their synthesis by monitoring grain development (Chapter VI corresponding to a scientific article in preparation).
5. To characterize a *H. chilense* collection for carotenoid content and esterification profile (Chapter VII corresponding to a scientific article in preparation).

Chapter I:

Cytological and molecular characterization of wheat-*Hordeum chilense* chromosome $7H^{ch}$ introgression lines

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Abstract

Chromosome 7H^{ch} of *Hordeum chilense* carries the *Phytoene synthase 1* (*Psy1*) gene encoding the first step in the carotenoid biosynthetic pathway. As such it can be used in the improvement of seed carotenoid content in wheat. However, its use in wheat breeding programs requires the availability of genetic stocks carrying this gene. In this work, four introgressions of chromosome 7H^{ch} into wheat have been characterized by in situ hybridization of labeled DNA probes and by several sets of DNA markers. Chromosome-specific SSR were used for the identification of wheat chromosomes. Besides 113 Conserved Orthologous Set (COS) markers were tested for homoeologous group 7, of which 97 amplified in *H. chilense* and 32 were polymorphic between *H. chilense* and wheat, and 28 EST (Expressed Sequence Tag) barley markers previously allocated to chromosome 7. A total of 60 markers (32 COS and 28 EST) were allocated to chromosome 7H^{ch} with 28 assigned to 7H^{ch}S and 22 to 7H^{ch}L. A combination of in situ probing and marker genotyping have shown that among the four introgressions there was a substitution line 7H^{ch}(7D), a ditelosomic addition line for the long arm of 7H^{ch} and two homozygous centric translocations 7H^{ch}S·2DS and 7H^{ch}S·5AL. The *Psy1* gene was localized on the short arm of 7H^{ch}. The positions of markers from the International Barley Consortium map (IBSC2012) were determined and the comparative arm location between *H. chilense* and *H. vulgare* is discussed. The genetic stocks characterized here include new wheat-*H. chilense* recombinations useful for genetic studies and with a potential for breeding.

This work has allowed the identification of several genetic stocks carrying *Psy1* from *H. chilense,* including two translocation lines (T7H^{ch}S·2DS y T7H^{ch}S·5AL). These translocations have the higher potential to be used as parental lines in wheat breeding. Besides, this work has identified new molecular markers useful for selecting new *H. chilense*-wheat introgressions. Finally, this work has corroborated the lack of collinearity between chromosomes 7H^{ch} from *H. chilense* and 7H from barley, which includes the position of *Psy1.*

Keywords: Wheat, *Hordeum chilense*, Introgression lines, Cytogenetics, FISH, Barley

Resumen

El gen Fitoeno sintasa 1 (*Psy1*, Phytoene synthase 1) que codifica el primer paso en la ruta de biosíntesis de carotenoides, se localiza en el cromosoma 7H^{ch} de *Hordeum chilense*. Este gen puede utilizarse en la mejora del contenido de carotenoides de semillas de trigo. Sin embargo, su utilización en programas de mejora de trigo requiere de la disponibilidad de stocks genéticos en los que se hayan introgresado este gen. En este trabajo se han caracterizado cuatro introgresiones del cromosoma 7H^{ch} en trigo mediante hibridación *in situ* con sondas de ADN marcadas y el uso de varios grupos de marcadores de ADN. Para la identificación de los cromosomas de trigo se han utilizado marcadores microsatélites cromosoma-específicos. Además, se han analizado 113 marcadores de tipo COS (por sus siglas en inglés: "Conserved Orthologous Set") correspondientes al grupo homeólogo 7, de trigo. De estos marcadores, 97 amplificaron en *H. chilense* y 32 fueron polimórficos entre *H. chilese* y trigo. También se han utilizado 28 marcadores de cebada de tipo EST (por sus siglas en inglés: "Expressed Sequence Tag") previamente localizados en el cromosoma 7. Un total de 60 marcadores (32 COS y 28 EST) se localizaron en el cromosoma 7H^{ch}, de los cuales 28 se asignaron al brazo corto y 22 al brazo largo. Los resultados obtenidos mediante hibridación *in situ* y el genotipado con marcadores moleculares ha mostrado que las introgresiones obtenidas corresponden a una línea de substitución de cromosómica 7D/7H^{ch}, una línea de adición ditelosómica del brazo largo del 7H^{ch} y dos líneas de translocación (7H^{ch}S·2DS y 7H^{ch}S·5AL). El gen *Psy1* se mapeo físicamente en el brazo corto del cromosoma 7H^{ch}. Además, se han determinado las posiciones relativas de los marcadores utilizando el "International Barley Consortium Map" (IBSC2012) y se discute la localización comparada de los marcadores entre *H. chilense* y *H. vulg*are.

Este trabajo ha permitido caracterizar nuevo germoplasma portador del gen *Psy1*, incluyendo dos translocaciones (T7H^{ch}S·2DS y T7H^{ch}S·5AL) que son las líneas con mayor potencial para su uso en programas de mejora genética de trigo. Además, este trabajo ha permitido identificar nuevos marcadores moleculares útiles para la selección de nuevas introgresiones de *H. chilense* en trigo. Así mismo, este trabajo ha permitido corroborar que las partes distales de los cromosomas 7H^{ch} (*H. chilense*) y 7H (cebada) no mantienen la colinealidad, lo que incluye la posición del gen *Psy1*.

Palabras claves: trigo, *Hordeum chilense*, líneas de introgresión, citogenética, FISH, cebada

Chapter II:

Physical mapping of *Psy1* gene in *Hordeum chilense* by FISH

Scientific article in preparation with copyright shared among authors: Mattera MG, Karafiátová M, Endo T, Atienza SG, Cabrera A, Doležel J.

Abstract

Phytoene synthase 1 (*Psy1*) gene is involving in the first step of carotenoid biosynthesis pathway. The potential of *Psy1* from *Hordeum chilense* to enhance the seed carotenoid content in wheat has been previously reported. *Psy1* is located on chromosome $7H^{ch}$ but its relative position is still unknown, since *Psy1* is mapped in a short linkage group which remains unlinked. In the present work, we developed physical mapping of the *Psy1* gene on $7H^{ch}$ chromosome by directly labelling FISH method. As result, *Psy1* was cytogenetically located at proximal position on the $7H^{ch}S$ arm in two *H. chilense* accessions. *Psy1* locus was mapped at position 0.21 ± 0.08 of fraction lengths on $7H^{ch}S$ arm.

Previous works using genetic stocks have proven that *Psy1* from *H. chilense* was located in the short arm of chromosome $7H^{ch}$. This constitutes a break of collinearity between *H. chilense* and wheat, where *Psy1* is located in the distal part of chromosome of homoeologous group 7. However, all *H. chilense*-wheat genetic stocks had been developed using a single *H. chilense* accession (H1) as donor. Thus, the possibility that the break of collinearity was exclusive to this genotype was an open question. Thus, our current results are relevant since they show that the break of collinearity for *Psy1* is not exclusive to H1.

Resumen

El gen Fitoene sintasa 1 (*Psy1*) está involucrado en el primer paso de la ruta de biosíntesis de carotenoides. Se ha descrito previamente el potencial del gen *Psy1* de *Hordeum chilense* para mejorar el contenido de carotenoides de las semillas. Este gen está localizado en el cromosoma $7H^{ch}$ pero su posición relativa es desconocida aún, ya que se encuentra mapeado en un grupo de ligamiento pequeño separado del mapa del cromosoma $7H^{ch.}$. En el presente trabajo, se ha llevado a cabo el mapeo físico del gen *Psy1* en el cromosoma $7H^{ch}$ mediante el método de FISH (por sus siglas en inglés "Fluorescence *in situ* Hybridization") usando el marcaje directo. Como resultado, el locus *Psy-1* se ha localizado a un 21% de FL (por sus siglas en inglés "fraction lengths") en la región proximal del brazo corto del cromosoma $7H^{ch}$.en dos accesiones de *H. chilense* (H1 y H7).

En trabajos previous se había demostrado que el gen *Psy1* se encontraba en el brazo corto del cromosoma $7H^{ch}$ de *H. chilense* lo que constituye una rotura de la colinealidad entre *H. chilense* y trigo. Sin embargo, estos resultados se habían conseguido gracias a la utilización de líneas desarrolladas a partir de un único genotipo de *H. chilense* (H1). Por tanto, cabía la posibilidad de que la ruptura de la colinealidad fuera exclusiva del genotipo H1. De ahí que los resultados de este capítulo sean relevantes, ya que muestran que el gen *Psy1* se encuentra en el brazo corto del cromosoma $7H^{ch}$ también en el genotipo H7 y, por tanto, la ruptura de la colinealidad no es exclusiva de un solo genotipo.

Chapter III:

Lutein esterification in wheat endosperm is controlled by the homoeologous group 7, and is increased by the simultaneous presence of chromosomes 7D and 7H^{ch} from *Hordeum chilense*

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Abstract

The high carotenoid content in tritordeum (×Tritordeum Ascherson et Graebner) grains is derived from its wild parent, *Hordeum chilense* Roem. et Schulz. *Phytoene synthase 1* is located in the chromosome $7H^{ch}S$ and plays a major role in this trait. This study investigates the impact of the introgression of chromosome $7H^{ch}$ into common wheat background on carotenoid composition, including xanthophylls esterified with fatty acids (monoesters and diesters). All the genetic stocks carrying *Psy1* from *H. chilense* increased their carotenoid content with respect to common wheat. Also, significant changes in the carotenoid profile were detected in different genetic stocks. The most relevant one was the increase in the content of lutein diesters when both $7H^{ch}$ and 7D were present which indicates the existence of genes involved in the esterification of xanthophylls in both chromosomes. Furthermore, our results suggest that $7H^{ch}$ genes preferentially esterify lutein with palmitic acid while 7D is either indifferent to the fatty acid or it prefers linoleic acid for lutein esterification. The involvement and complementarity of $7H^{ch}$ and 7D are highly significant considering the scarcity of previous results on lutein esterification in wheat. In addition our results demonstrate that *Psy1* from *H. chilense* is an interesting source for the enhancement of carotenoid content in grains of wheat. Indeed, previous studies have been performed using chromosome addition lines, and thus, *Psy1* from *H. chilense* was in the presence of all the wheat *Psy1* homoeologues. However, our current results with chromosome substitution lines are significant since these lines lacks one of the wheat native *Psy1* but they show a superior carotenoid content in grains.

Additional keywords: alien Triticeae; carotenoid esters; esterification; genetic stocks; lutein esters; yellow pigment content

Resumen

El alto contenido de carotenoides en los granos de tritordeum (×Tritordeum Ascherson et Graebner) se debe a su parental silvestre, *Hordeum chilense* Roem. et Schulz. El gen *Fitoeno sintasa 1* (*Psy1*) está localizado en el brazo corto del cromosoma $7H^{ch}$ y tiene un papel principal en este carácter. Este estudio investiga la influencia de las introgresiones del cromosoma $7H^{ch}$ dentro del fondo genético del trigo común sobre la composición de carotenoides, incluyendo las xantofilas esterificadas con ácidos grasos (monoésteres y diesters). Todos los genotipos portadores del *Psy1* de *H. chilense* incrementaron su contenido en carotenoides con respecto a el trigo harinero. También, se detectaron cambios significativos en el perfil de carotenoides en los distintos genotipos. El más relevante fue el incremento en el contenido de diésteres de luteína cuando ambos cromosomas $7H^{ch}$ y 7D estaban presentes, lo cual indica la existencia de genes involucrados en la esterificación de las xantofilas en ambos cromosomas. Además, los resultados obtenidos sugieren que los genes del cromosoma $7H^{ch}$ esterifican preferencialmente la luteína con ácido palmítico mientras que el cromosoma 7D es indiferente al ácido graso o incluso prefiere el ácido linoléico para la esterificación de la luteína. La participación y complementariedad de los cromosomas $7H^{ch}$ and 7D es altamente significativa considerando la escasez de resultados previos en la esterificación de la luteína. Además, nuestros resultados demuestran que el gen *Psy1* de *H. chilense* es interesante para la mejora del contenido en carotenoides en granos de trigo. De hecho, los estudios previous realizados hasta el momento se habían desarrollado utilizando líneas de adición y, por tanto, el gen *Psy1* de *H. chilense* estaba en presencia de los 3 genes homeólogos de trigo. Por tanto, se estaba observando el efecto de la adición de este gen. Sin embargo, su utilización en mejora requiere el desarrollo de líneas euploides lo que implica la sustitución de uno de los genes de trigo por el de *H. chilense*. Por tanto, resultaba necesario estudiar el efecto de sustitución de este gen. Nuestros resultados con líneas de sustitución cromosómicas son relevantes en este sentido, ya que demuestran que el gen *Psy1* de *H. chilense* incrementa el contenido en carotenoides en grano de trigo con independencia del homeólogo de trigo al que sustituya.

Palabras claves: Triticeae, ésteres de carotenoides, esterificación, recursos genéticos, contenido de pigmento amarilo

Chapter IV:

Characterization of a set of common wheat-*Hordeum chilense* chromosome 7H^{ch} introgression lines and its potential use in research on grain quality traits

Published as:

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Abstract

Chromosome $7H^{ch}$ from *Hordeum chilense* has potential for improving seed carotenoid content in wheat since it carries a Phytoene synthase 1 (*Psy1*) gene, which has a major role in this trait. Structural changes in chromosome $7H^{ch}$ were obtained in common wheat background by crossing the wheat disomic substitution line $7H^{ch}$(7D) with a disomic addition line carrying chromosome $2C^{c}$ from *Aegilops cylindrica* in common wheat cv. 'Chinese Spring'. Rearranged $7H^{ch}$ chromosomes were cytologically characterized by FISH. A set of 24 molecular markers and the *Psy1* gene were used to identify the *H. chilense* chromosome segments involved in the introgressions. Six structural rearrangements of chromosome $7H^{ch}$ were identified. They included three homozygous wheat-*H. chilense* centromeric translocations; one involving the $7H^{ch}S$ arm (T-$7H^{ch}S$·A/B) and two involving the $7H^{ch}L$ arm (T1-$7H^{ch}L$·A/B and T2-$7H^{ch}L$·A/B). In addition, one $7H^{ch}S$ arm deletion, one $7H^{ch}L$·$7H^{ch}L$ isochromosome and one $7H^{ch}S$ telosome were obtained in hemizygous condition. These genetic stocks will be useful for studying the effect of chromosome $7H^{ch}$ on wheat flour colour.

Keywords: chromosome structure, cytogenetic stocks, grain colour, molecular markers, *Triticum aestivum*.

Resumen

El cromosoma 7H^{ch} de *Hordeum chilense* tiene potencial para la mejora del contenido de carotenoides de las semillas en trigo dado que este cromosoma lleva el gen Fitoeno sintasa 1 (*Psy1*), el cual tiene un rol principal en este carácter. En este trabajo se utilizado la acción gametocida del cromosoma 2C de *Aegilops cylindrica* para inducir nuevos cambios estructurales del cromosoma 7H^{ch} en el fondo genético de trigo. Para ello, se ha realizado el cruzamiento entre la línea de substitución disómica 7H^{ch}(7D) con la línea de adición disómica del cromosoma 2C^{c} de *Aegilops cylindrica* en el fondo genético del cultivar de trigo común "Chinese Spring". La descendencia obtenida se ha evaluado citológicamente mediante FISH (Fluorescent *In Situ* Hibridization) y se han seleccionado aquellas plantas que contienen cambios estructurales en el cromosoma 7H^{ch}. Se han utilizado 24 marcadores moleculares y el gen *Psy1* para determinar el fragmento del cromosoma 7H^{ch} implicado en las introgresiones. Los resultados obtenidos muestran seis reordenamientos estructurales del cromosoma 7H^{ch}: tres de ellos consisten en translocaciones homocigóticas trigo-*H. chilense*, una involucrando el brazo corto (T-7H^{ch}S•A/B) y dos al brazo largo (T1-7H^{ch}L•A/B y T2-7H^{ch}L•A/B). Además, se han obtenido en hemicigosis, una deleción del brazo corto del 7H^{ch}, un isocromosoma del brazo largo del 7H^{ch} (7H^{ch}L•7H^{ch}L) y un telosoma del brazo corto del 7H^{ch}. El germoplasma obtenido y caracterizado puede ser útil para estudiar el efecto del cromosoma 7H^{ch} sobre el color de la harina de trigo.

Palabras claves: estructura cromosómica, recursos genéticos, color del grano, marcadores moleculares, *Triticum aestivum*.

Chapter V:

Lutein ester profile in wheat and tritordeum can be modulated by temperature: Evidences for regioselectivity and fatty acid preferential of enzymes encoded by genes on chromosomes 7D and 7H^{ch}

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Mattera MG, Hornero-Méndez, Atienza SG (2017). Lutein ester profile in wheat and tritordeum can be modulated by temperature: Evidences for regioselectivity and fatty acid preferential of enzymes encoded by genes on chromosomes 7D and 7H^{ch}. *Food Chemistry* 219, 199–206.

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Abstract

The increase of lutein retention through the food chain is desirable for wheat breeding. Lutein esters are more stable than free lutein during post-harvest storage and two loci on chromosomes 7D and $7H^{ch}$ are important for esterification. We investigated the effect of temperature during grain filling on carotenoid accumulation and lutein ester profile including fatty acid selectivity (palmitic *vs*. linoleic) and regioselectivity (esterification at positions 3 *vs.* 3'). Three different temperature regimes were assayed (controlled, semi-controlled and non-controlled). A high temperature regime during grain filling results in lower total carotenoid and free lutein contents. However, lutein esters did not show significant variations which prove that they are more stable than free carotenoids *in vivo.* Our results also showed the enzymes encoded by chromosomes $7H^{ch}$ and 7D are complementary. Indeed, they show differential preferences for the fatty acid (palmitic and linoleic, respectively) and regioselectivity (3 and 3', respectively). Besides, *H. chilense* has additional genes for esterification. Finally, the increase of temperature favoured the accumulation of lutein esters with linoleic acid and the synthesis of regioisomers at position 3'.

The higher *in vivo* stability of lutein esters suggests that a higher esterification capacity would be desirable to increase lutein retention for the production of wheat-derived products such as pasta, yellow-alkaline noodes or high-lutein bread. Besides, the complementarity observed between 7D and $7H^{ch}$ may explain the higher levels of lutein diesters detected when both chromsomes are simultaneously present. In any case, tritordeums lines synthesize higher amounts of lutein esters which indicates that *H. chilense* genome possesses other genes important for lutein esterification.

Keywords: Carotenoid, esterification, *Hordeum chilense,* lutein, lutein acylesters regioisomers, tritordeum, wheat

Resumen

El incremento de la retención de la luteína a través de la cadena alimenticia es deseable para la mejora de trigo. Los ésteres de luteína son más estables que las luteínas libres durante el almacenaje del grano y dos *loci* ubicados en los cromosomas 7D y 7H^{ch} son importantes para la esterificación. En este trabajo hemos investigado el efecto de la temperatura durante el llenado del grano sobre la acumulación de carotenoides y el perfil de ésteres de luteína incluyendo la selectividad por el ácido graso (palmítico *vs.* linoleico) y la regio-selectividad (esterificación en posición 3 *vs.* 3'). Se desarrollaron tres ensayos de campo con distintos regímenes de temperatura (controlada, semi-controlada y no controlada) a partir del espigado. La incidencia de altas temperaturas durante el desarrollo del grano produjo una disminución en el contenido total de carotenoides y de luteína libre. Sin embargo, los niveles de ésteres de luteína no mostraron una variación significativa lo que indica que son más estables que los carotenoides *in* vivo. Nuestros resultados también indican que las enzimas codificadas por los cromosomas 7D y 7H^{ch} son complementarias. De hecho, estas enzimas difieren tanto en el ácido graso (palmítico y linoleico, respectivamente) que esterifican de forma preferencial, como en la posición en la que lo esterifican (regioselectividad, posiciones 3 y 3', respectivamente). Además, *H. chilense* tiene genes adicionales para esterificación. Finalmente, el incremento de la temperatura favorece la acumulación de los ésteres de luteína con ácido linoleico así como la síntesis de regioisómeros en la posición 3'.

La mayor estabilidad *in vivo* de los ésteres de luteína sugiere que una mayor capacidad de esterificación sería deseable para incrementar la retención de luteína en la producción de diversos productos como pasta, fideos chinos alcalinos (amarillos) o pan con alto contenido en luteína. Además, la complementariedad observada entre los cromosomas 7D y 7H^{ch} podría explicar los altos niveles de diésteres de luteína observados cuando ambos cromosomas están presentes de forma simultánea. Por último, nuestros datos sugieren la existencia de genes adicionales para esterificación en *H. chilense*, ya que los tritórdeos muestran aún mayores niveles de ésteres de luteína que las líneas de sustitución.

Palabras claves: carotenoides, *Hordeum chilense,* luteína, regioisómeros acilésteres de luteína, tritordeum, trigo.

Chapter VI:

Carotenoid accumulation patterns and lutein esterification process during grain development.

Scientific article in preparation with copyright shared among authors: Mattera MG, Hornero-Méndez D, Atienza SG.

Abstract

Carotenoid esterification with fatty acids increases its lipid solubility and is considered a common means to sequester them into lipid-rich plastoglobules. Several studies have addressed the changes on lutein esterification profile in wheat and related cereals during seed storage. However, the moment when these compounds begin to form was not determined until now. A detailed analysis of carotenoid accumulation including lutein esterification profile as well the evolution on chlorophyll (a and b) contents was carried out during grain development in a set of wheat- *H. chilense* disomic substitution, durum and common wheat and tritordeum. The identified carotenoids included 9'-cis-neoxanthin, violaxanthin, all-trans-antheraxanthin, all-trans-zeaxanthin, all-trans-beta-carotene and lutein, which was the major carotenoid in all stages. A decreasing trend was observed for all identified pigments in the studied genotypes, which could be caused by both the increase in grain size hilling and the decrease of photosynthesis in the grain leading to pigment degradation. In addition, no lutein esters were detected before 32 dap. At 36 dap, lutein monoesters were detected in DS 7H^{ch}(7A) and DS 7H^{ch}(7B) while no lutein monoesters were detected in DS 7H^{ch}(7D) and Chinese Spring. Our results suggest that the coexistence of chromosomes 7D and 7Hch not only increases the proportion of lutein esterification but they also promote an earlier esterification. Finally, we found diversity for esterification ability in *H. chilense* since HT630 showed higher amounts of lutein esters than both HT621 and HT609.

Resumen

La esterificación de los carotenoides con ácidos grasos incrementa su lipo-solubilidad y es considerada un medio común para secuestrarlos en plastoglóbulos ricos en lípidos. Varios estudios han abordado los cambios en el perfil de esterificación de la luteína en trigo y otros cereales relacionados durante el almacenaje de semillas. Sin embargo, se desconoce el momento en que estos compuestos empiezan a formarse. De ahí que se abordara el estudio de la acumulación de carotenoides en grano, incluyendo tanto el perfil de esterificación de la luteína como la evolución en los contenidos de clorofilas (a y b), durante el desarrollo del grano en líneas de substitución de trigo-*H. chilense*, en trigo duro y harinero y en tritordeo. Los carotenoides identificados incluyeron 9'-*cis*-neoxanteno, violaxanteno, all-*trans*-anteraxanteno, all-*trans*-zeaxanteno, all-*trans*-beta-caroteno y luteína, la cual fue el principal carotenoide en todos los estadíos. Se observó una tendencia decreciente para todos los pigmentos en los genotipos estudiados, lo cual puede ser causado tanto por el incremento en el tamaño del grano como por la disminución de la fotosíntesis en el grano que conduce a la degradación de pigmentos. No se detectaron ésteres de luteína antes de los 36 dpa (días post antesis). Se detectaron monoésteres de luteína en las líneas de sustitución DS 7H^{ch}(7A) y DS 7H^{ch}(7B) a los 36 dpa lo que contrasta con los resultados obtenidos en la línea de sustitución DS 7H^{ch}(7D) y "*Chinese Spring"* (trigo común usado como control), donde no se detectó la presencia de monoésteres. Nuestros resultados sugieren que la coexistencia de los cromosomas 7D and 7H^{ch} no solo incrementa la proporción de ésteres de luteína sino también promueve una esterificación más temprana. Finalmente, los resultados obtenidos sugieren la existencia de diversidad para la habilidad de esterificación en *H. chilense* dado que la línea de tritórdeo HT630 mostró mayor contenido de ésteres de luteína que las líneas de tritórdeo HT621 y HT609.

Chapter VII:

Variability in carotenoid content and esterification profile in a collection of *Hordeum chilense*

Data included in a scientific article in preparation Atienza et al. Copyright shared among authors:

Abstract

Lutein degradation could be significant during grain and flour storage and food processing. Since lutein esters are more stable than free lutein, esterification may be important to increase carotenoid retention through the food chain. Despite the potential importance of lutein esterification, the knowledge of the genetic basis of esterification profile in cereals is scarce. *H. chilense* has been proposed as a good genetic model for the study of carotenoids in the Triticeae and it has potential for wheat breeding. This species is responsible for the high carotenoid content and the distinctive esterification profile found in tritordeum grains. In this study, we analyzed the germplasm collection of *H. chilense* for carotenoid content and esterification profile. This species showed a wide range of variation for carotenoid content (5.81 – 43.06 µg/g dry weight). Moreover, it exhibited a wide diversity for lutein esters indicating that this collection is an adequate diversity panel for further genetic studies. Besides, our results suggest that *H. chilense* has a preference for palmitic acid but that it is genotype-specific and not genome-specific.

Resumen

La degradación de la luteína puede ser significativa tanto durante el almacenaje de grano y harina como durante el procesamiento de alimentos. La esterificación puede ser importante para incrementar la retención de carotenoides a través de la cadena alimentaria dado que los ésteres de luteína son más estables que la luteína libre. A pesar de la importancia potencial de la esterificación de la luteína, el conocimiento de la base genética del perfil de esterificación es escaso. La especie *Hordeum chilense* ha sido propuesta como un buen modelo genético para el estudio de carotenoides en las Triticeae. Esta especie es responsable del alto contenido de carotenoides y del perfil de esterificación distintivo encontrado en el grano de tritórdeo. En este estudio, analizamos una colección de germoplasma de *H. chilense* para el contenido de carotenoides y el perfil de esterificación. Esta especie mostró una amplio rango de variación para el contenido de carotenoides (5.81 – 43.06 µg/g material seca). También exhibió una amplia diversidad para el contenido en ésteres de luteína, lo que sugiere que esta colección constituye un panel de diversidad adecuado para el desarrollo de estudios genéticos. Además, nuestros resultados sugieren que *H. chilense* tiene preferencia por el ácido palmítico pero esta es específica del genotipo y no del genoma.

General Discussion

Carotenoid pigments, mainly lutein, are the main responsible for the yellow colour of wheat and related cereals seeds. *H. chilense* has genes for the enhancement of carotenoid content in seeds (reviewed by Rodríguez-Suárez *et al.* 2010). This characteristic is interesting for tritordeum breeding where lutein content is one of its main quality attributes. In addition, the genes from *H. chilense* have also potential for wheat breeding. Indeed, a bright yellow colour is required for pasta production (Troccoli *et al.* 2000). Therefore durum wheat varieties have been selected for high yellow pigment content over the last decades (reviewed by Ficco *et al.* 2014). Similarly, new bread types based in yellowish flours are being produced from einkorn (*T. monococcum* L.) (Abdel-Aal *et al.* 2002) and tritordeum (×Tritordeum Aschers. et Graeb.) (Vivagram®, www.agrasys.es). The importance of carotenoids is not restricted to food colour. On the contrary, carotenoid-rich daily intakes have been associated with a reduced risk of certain diseases (reviewed by Rao and Rao 2007). Since cereals are staple foods, they can be considered ideal elements for use in biofortification strategies for functional food production (Bai *et al.* 2011).

The potential of wild relatives for the improvement of carotenoid content has been clearly evidenced in bread (Zhang *et al.* 2005) and durum wheat (Ceoloni *et al.* 2014) through the development of introgressions involving the homoeologous group 7. Collectively, previous studies have been shown that the wild barley *H. chilense* constitutes an important genetic pool for the increase of the yellow pigment contents (YPC) in wheat (Alvarez *et al.* 1994; Atienza *et al*. 2004; Rodríguez-Suárez *et al.* 2010). However, the use of *H. chilense* in wheat breeding requires the development of *H. chilense* introgressions into wheat. Many chromosomes of *H. chilense* carry genes related to carotenoid content and seed colour (Rodríguez-Suárez and Atienza 2012; 2014). All these regions have potential for wheat breeding but it is widely accepted that loci mapped on chromosomes of the homoeologous group 7 are the main responsible for this trait in wheat and related species of Triticeae tribe (Atienza *et al.* 2007a; Elouafi *et al.* 2001; Parker *et al.* 1998; Patil *et al.* 2008; Zhang *et al.* 2008). In particular, variations in the gene *Psy-1* is associated with the main differences in seed carotenoid content (Rodríguez *et al.* 2010; Ficcó *et al.* 2014). Accordingly, during this thesis we focused on the development of introgressions of chromosome $7H^{ch}$ into common wheat using the gametocidal system developed by Endo et al (1988). The gametocidal chromosome $2C^{c}$ from *Ae. cylindrica* was used to induce chromosome breaks on $7H^{ch}$ chromosome in common wheat background. Breaks were induced both at the centromere and interstitial regions of the chromosomes lending to the obtention of $7H^{ch}$ telosomes, wheat-$7H^{ch}$ translocations and $7H^{ch}$ terminal deletions. In particular, we developed and characterized seven stable and fertile lines and another three lines in hemizygous conditions (chapters I and IV, this thesis, Mattera *et al.* 2015a, Mattera and Cabrera 2017). The first group includes: a wheat- *H. chilense* disomic substitution line of 7D chromosomes by $7H^{ch}$ [DS $7H^{ch}$(7D)], a wheat- *H. chilense* ditelosomic addition line of $7H^{ch}$L [Dt $7H^{ch}$L] and five wheat- *H. chilense* homozygous centric translocations, corresponding three to the short arm of $7H^{ch}$ [T-$7H^{ch}$S·7AL, T-$7H^{ch}$S·7DL and T-$7H^{ch}$S·A/B] and two to the long arm [T1-$7H^{ch}$L·A/B and T2-$7H^{ch}$L·A/B]. The second group includes those line carrying a deletion of $7H^{ch}$S [Del $7H^{ch}$S], an isochromosome $7H^{ch}$L·$7H^{ch}$L [Iso $7H^{ch}$L]

and a telosome 7H^{ch}S [Telo 7H^{ch}S] at hemizygous condition. All these genetic stocks were cytological characterized by FISH/GISH using both pAs1 and *H. chilens*e genomic DNA as probes. In addition, a molecular characterization of these stocks was carried out using polymorphic markers (COS and EST markers) between wheat and *H. chilense*. Transferences of a set of COS markers to 7H^{ch} chromosome was obtained, increasing the number of specific molecular markers for this chromosome. *Psy-1* gene was also used to genotyping these lines and we confirmed its presence in DS 7H^{ch}(7D), T-7H^{ch}S·7AL, T-7H^{ch}S·7DL, T-7H^{ch}S·A/B and Telo 7H^{ch}S lines. Accordingly, all the genetic stocks developed in this thesis suppose a first key step towards the use of *Psy-1* gene from *H. chilense* in wheat breeding. The development of wheat- 7H^{ch} translocation lines opens interesting possibilities to wheat improvement, since these could allow the combinations of orthologous *Psy-1* genes into wheat genetic background and they also could serve to study the synergic effect on carotenoid content. So far, two wheat- *H. chilense* translocation lines involving short and long chromosome arms were obtaining by use of *ph1b* background (Rey *et al.* 2015).

Overall, the arm locations of the molecular markers have showed a good correspondence with those found in common barley; however we have detected a collinearity break in the distal portion of both arms of 7H^{ch} chromosome. The location of *Psy-1* gene is one of these notorious changes. We mapped this gene on the short arm of 7H^{ch} whereas it has been mapped on distal portion of the long arm of the homoeologous group 7 chromosomes in wheat and barley (rewieved by Ficco *et al.* 2014; Rodríguez-Suárez *et al.* 2011). Previous studies have also reported collinearity breaks between common and wild barley (Hagras *et al.* 2005; Said and Cabrera 2009). Our results suggest the occurrence of an inversion involving both the distal part of short and long arms of 7H^{ch} chromosome.

All the wheat-*H. chilense* genetic stocks carrying the *Psy-1* gene have been developed using a single *H. chilense* genotype (H1) as donor. Thus, we not discard the possibility that the inversion mentioned above was limited to H1 genotype. A short-stay visit at Centre of Plant Structural and Functional Genomics of the Institute of Experimental Botany in Czech Republic allowed the physical map of *Psy1* in both H1 and H7 genotypes (Chapter II, this thesis, Mattera *et al.* in preparation). Our results suggest that the collinearity break is not exclusive of one accession since *Psy1* was located on the same position in both H1 and H7 accessions.

Before the beginning of this thesis, the potential of *H. chilense* for the improvement of carotenoid/lutein content in wheat has been shown using chromosome addition lines (Alvarez *et al.* 1998) or from data obtained in tritordeum (Atienza *et al.* 2007b; Rodríguez-Suárez *et al.* 2014). However, the transference of a chromosome or a chromosome region from *H. chilense* to wheat usually implies that the homoeologous region from wheat is loss in the process. Thus, a substitution effect is produced. The transference of *Psy1* from *H. chilense* to wheat would imply the loss of either *Psy1-7A, Psy1-7B* or *Psy1-7D* from homoeologous chromosomes. We have evaluated the substitution effect among homoeologous group 7 of wheat and chromosome 7H^{ch} for carotenoid content (chapter III, this thesis, Mattera *et al.* 2015b). Our results showed that all the lines carrying *Psy-1* gene from *H. chilense* exhibited higher

carotenoid and lutein concentration than bread wheat. On the contrary, the presence of *Psy2* or *Psy3*, located on chromosome 5H^{ch}, did not increase seed carotenoid content which confirms the role of *Psy1* on carotenogenesis during grain filling.

Lutein is found in free form or esterified with fatty acids. Esters with linoleic and palmitic acids were the only lutein esters detected in agreement with other studies (Ahmad *et al.* 2013; Mellado-Ortega and Hornero-Mendez 2012; Mellado-Ortega and Hornero-Méndez 2015). On the contrary, lutein esters with oleic or linolenic acids were not detected although they have been identified in bread wheat, einkorn and emmer (Ziegler *et al.* 2015). In addition, our results showed that the wheat-*H. chilense* chromosome substitution lines were not equivalent for the lutein esterification profile. Indeed, the simultaneous presence of both 7H^{ch} and 7D resulted in higher amounts of lutein diesters. Our results indicate the existence of at least one locus on the 7H^{ch} codifying an enzyme for lutein esterification and we also confirmed the existence of a locus located on chromosome 7D as has been previously reported (Ahmad *et al.* 2013). Besides, we showed that lutein esterification is not proportional to total lutein content since the proportion of lutein diesters in bread wheat is higher than in other genotypes with higher lutein content. Our results also indicate that esterification enzymes codifying by 7H^{ch} and 7D chromosomes seem to have complementary activity. Indeed, specificity to the substrate involved in the esterification was revealed, since *H. chilense* genome promotes the lutein esterification with palmitic acid on the β-ring of lutein, in agreement with previous studies (Mellado-Ortega and Hornero-Méndez 2015); whereas the affinity of 7D is toward the linoleic acid or even is indifferent to the fatty acid used. In addition, the simultaneous presence of both 7H^{ch} and 7D chromosomes results in earlier formation of lutein esters (Chapter VI). The detailed analysis of pigment accumulation during grain development showed that no lutein esters were produced until the final stages of grain filling. However, lutein esters were produced earlier when both 7H^{ch} and 7D are present, which constitutes further proof of their complementarity. In addition, the differences among tritordeums in the esterification profile suggest the existence of genetic diversity in *H. chilense.*

Lutein esterification is important to improve carotenoid retention through the food chain. Several studies have revealed the direct implication of esterification on lutein stability and conservation in wheat and related cereals during seed storage (Ahmad *et al.* 2013; Doblado-Maldonado *et al.* 2012; Kaneko and Oyanagi 1995; Mellado-Ortega and Hornero-Mendez 2012; Mellado-Ortega and Hornero-Méndez 2016a b). Environmental factors, such as temperature, need to be considered since they are important for lutein stability. Several studies have confirmed that the esterification process is modulated by the temperature during grain storage and when thermal conditions were more intense (~37 ºC), an intensification of *de novo* lutein esterification was observed in tritordeum resulting in higher lutein retention (Ahmad *et al.* 2013; Mellado-Ortega and Hornero-Méndez *et al.* 2016a b; Mellado-Ortega *et al.* 2015). Overall, carotenoids followed a temperature-dependent degradative pattern during both short and long- storage period. Nevertheless, the role of esterification was corroborated as mechanism that provides extended stability to lutein, since lutein ester are more stable than free molecules with increase of storage temperature (Subagio *et al.* 1999, Ahmad et al 2013, Mellado-Ortega and Hornero-Méndez et al 2016a). Considering

the importance of temperature during grain storage on both lutein stability and esterification, we studied the effect of temperature during grain filling on carotenoid accumulation and lutein ester profile (chapter V, this thesis, Mattera *et al.* 2017). Our results showed that lutein esters were more stable than free carotenoids *in vivo* and that the increase of temperature favoured the accumulation of lutein esters with linoleic acid and the synthesis of regioisomers at position 3'. Besides, our results also confirmed the complementarity between 7D and $7H^{ch}$ chromosomes. Indeed, they show differential preferences for the fatty acid (linoleic and palmitic, respectively) and regioselectivity (3' and 3, respectively) have been found which constitutes an interesting result for further genetic studies.

Despite the advances described above, the genetic basis of lutein esterification in wheat and related cereals is still poorly understood. The first QTL for lutein esterification was located on 2B chromosome (Howitt *et al.* 2009), but these results have not been further confirmed. The high carotenoid content and it distinctive lutein esterification profile suggest that *H. chilense* may be a good model to study the genetic bases of lutein esterification. In this thesis we characterized a germplasm collection of *H. chilense* for carotenoid content and lutein ester profile (chapter VII, this thesis). The wide range of variation observed and the differences in the lutein ester profile confirm that this collection is a suitable diversity panel for genetic studies.

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CONCLUSIONS

1. A set of wheat-*H. chilense* introgression lines have been obtained. The developed and characterized genetic stocks include addition, subtititution, deletion and translocation lines of chromosome 7H^{ch} in common wheat background (derived from: **Chapter I** corresponding to published article: 'Cytological and molecular characterization of wheat-*Hordeum chilense* chromosome 7H^{ch} introgression lines', Mattera *et al.* 2015; and **Chapter IV** corresponding to published article: 'Characterization of a set of common wheat-*Hordeum chilense* chromosome 7H^{ch} introgression lines and its potential use in research on grain quality traits', Mattera MG and Cabrera A. 2017).
2. Molecular characterization of the these genetic stocks allowed identifying a good collinearity between homoeologous chromosome 7 in both *H. chilense* and *H. vulgare* with the exception of an inversion affecting the distal end of both chromosome arms, including the *Psy-1* gene. This inversion seems to be not exclusive of one accession of *H. chilense* (derived from: **Chapter I** corresponding to published article: 'Cytological and molecular characterization of wheat-*Hordeum chilense* chromosome 7H^{ch} introgression lines', Mattera *et al.* 2015; and **Chapter II** corresponding to a scientific article in preparation).
3. Introgression of *Psy-1* gene from *H. chilense* into common wheat background enhances seed carotenoid content. On contrary, neither *Psy-2* and *Psy-3* from *H. chilense* produced this effect (derived from: **Chapter III** corresponding to published article: 'Lutein esterification in wheat endosperm is controlled by the homoeologous group 7, and is increased by the simultaneous presence of chromosomes 7D and 7H^{ch} from *Hordeum chilense'*, Mattera *et al.* 2015).
4. Wheat-*H. chilense* chromosome substitution lines involving homoeologous chromosome 7 are not equivalent for lutein esterification profile. Simultaneous presence of both 7H^{ch} and 7D chromosomes results in higher amount of lutein diesters, which confirms the existence of a locus for lutein esterification on 7D. Besides, there is at least one locus for lutein esterification on 7H^{ch} (derived from: **Chapter III** corresponding to published article: 'Lutein esterification in wheat endosperm is controlled by the homoeologous group 7, and is increased by the simultaneous presence of chromosomes 7D and 7H^{ch} from *Hordeum chilense'*, Mattera *et al.* 2015).
5. Lutein esterification is not proportional to total lutein content (derived from: **Chapter III** corresponding to published article: 'Lutein esterification in wheat endosperm is controlled by the homoeologous group 7, and is increased by the simultaneous presence of chromosomes 7D and 7H^{ch} from *Hordeum chilense'*, Mattera *et al.* 2015).
6. In the genetic stocks analyzed in this thesis, the esterification enzymes codified by genes located on 7H^{ch} and 7D chromosomes show complementary activity. The *H. chilense* genotype H1 promotes lutein esterification with palmitic acid on the β-ring of lutein whereas 7D is either indifferent or it prefers linoleic acid for lutein esterification (derived from: **Chapter III** corresponding to published article: 'Lutein esterification in wheat endosperm is controlled by the

homoeologous group 7, and is increased by the simultaneous presence of chromosomes 7D and 7H^{ch} from *Hordeum chilense'*, Mattera *et al.* 2015).

7. Temperature regime during grain development has a modulating effect on carotenoid content and lutein esterification. Lutein esters are more stable than free carotenoids *in vivo*. In addition, the increase of temperature promotes the accumulation of lutein esters with linoleic acid and the synthesis of regioisomers at position 3´ constituting further proof of complementarity among chromosomes 7D and 7H^{ch} (derived from: **Chapter V** corresponding to published article: 'Lutein ester profile in wheat and tritordeum can be modulated by temperature: Evidences for regioselectivity and fatty acid preferential of enzymes encoded by genes on chromosomes 7D and 7H^{ch}', Mattera *et al.* 2017).
8. The simultaneous presence of 7D and 7H^{ch} results in earlier formation of lutein esters during grain filling but not lutein esters are produced before 36 days post anthesis (derived from: **Chapter VI** corresponding to a scientific article in preparation).
9. The collection of *H. chilense* germplasm shows a high diversity for carotenoid content and lutein esterification profile and so, it constitutes a suitable panel for genetic studies (derived from: **Chapter VII** corresponding to a scientific article in preparation).