1 **This is a postprint of the article by** M.G. Mattera, A. Cabrera, D. Hornero-Méndez and S.G.
2 Atienza. Lutein esterification in wheat endosperm is controlled by the homoeologous group 7, and
3 is increased by the simultaneous presence of chromosomes 7D and 7H^{ch} from *Hordeum*
4 *chilense*. ***Crop & Pasture Science* 2015, 66, 912-921.**

5
6 The definitive version of this article is available on the journal's website.

7 <http://dx.doi.org/10.1071/CP15091>

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*

M.G. Mattera^{A,B}, Cabrera A^B, D. Hornero-Méndez^C, S.G. Atienza^{A,D}

^A*Institute for Sustainable Agriculture, CSIC, E-14004 Córdoba, Spain*

^B*Department of Genetics, ETSIAM, University of Córdoba, Campus de Excelencia Internacional Agroalimentario, ceiA3, 14071 Córdoba, Spain.*

^C*Departament of Food Phytochemistry, Instituto de la Grasa (CSIC). Campus Universidad Pablo de Olavide, Edificio 46. Ctra. de Utrera, Km 1, E-41013 Sevilla, Spain.*

^DCorresponding author. Email: sgatienza@ias.csic.es

34

35 **Abstract**

36 The high carotenoid content in tritordeum (×Tritordeum Ascherson et Graebner) grains is derived from its
37 wild parent, *Hordeum chilense* Roem. et Schulz. *Phytoene synthase 1* is located in the chromosome
38 7H^{ch}S and plays a major role in this trait. This study investigates the impact of the introgression of
39 chromosome 7H^{ch} into common wheat background on carotenoid composition, including xanthophylls
40 esterified with fatty acids (monoesters and diesters). All the genetic stocks carrying *Psy1* from *H. chilense*
41 increased their carotenoid content with respect to common wheat. Also, significant changes in the
42 carotenoid profile were detected in different genetic stocks. The most relevant one was the increase in the
43 content of lutein diesters when both 7H^{ch} and 7D were present which indicates the existence of genes
44 involved in the esterification of xanthophylls in both chromosomes. Furthermore, our results suggest that
45 7H^{ch} genes preferentially esterify lutein with palmitic acid while 7D is either indifferent to the fatty acid or it
46 prefers linoleic acid for lutein esterification. The involvement and complementarity of 7H^{ch} and 7D are
47 highly significant considering the scarcity of previous results on lutein esterification in wheat.

48

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

51

Introduction

Endosperm color of wheat grains, which is mainly due to carotenoid accumulation, is an important quality criterion in breeding programs. Over the last few decades, durum wheat (*Triticum turgidum* spp. *durum*) has been selected for high yellow pigment content (YPC) since a bright yellow color is demanded for pasta production (Ficco *et al.* 2014). White flour is traditionally demanded by consumers for the consumption of bread. However, new bread types based on yellowish flours are being produced from einkorn (*Triticum monococcum* L.) (Abdel-Aal *et al.* 2002). Similarly, the commercialization of the bread from tritordeum (*×Tritordeum* Aschers. et Graeb.) (Vivagram®, www.agrasys.es) is also based on yellowish flours since the yellow color constitutes an interesting and distinctive characteristic for the diversification of this bread with respect to those from common wheat [reviewed by Rodríguez-Suárez *et al.* 2010)]. Moreover, the yellow color could be an interesting target for common wheat breeding since the creamy color of bread wheat flour partly contributes to the bright yellow color of yellow alkaline noodles (YAN) (Mares and Campbell 2001).

Carotenoids play essential roles in plants including light-harvesting, protection against oxidative damage, and photo-protection among others (Cuttriss *et al.* 2011), and they are present in many parts of the plants (Britton and Hornero-Mendez 1997). Carotenoids can only be synthesized *de novo* by plants, certain bacteria, and fungi but they play important roles for human health since they show important biological activities when ingested (Fernández-García *et al.* 2012). Further, the consumption of carotenoid-rich diets has been associated with a reduced risk of certain diseases. For instance, carotenoids with provitamin A activity, such as β -carotene, are important targets in the alleviation of vitamin A deficiency, which is one of the major health concerns in developing countries (WHO 2009). Similarly, the biological functions of carotenoids include defense against reactive oxygen species, inhibition of carcinogenesis and a lower risk of developing cardiovascular diseases [reviewed by Britton *et al.* (2009)]. In addition, epidemiological studies have shown an inverse correlation between the progression of age-related macular degradation and the intake of lutein and zeaxanthin rich-vegetables (Landrum and Bone 2004). Although cereals have a low carotenoid content compared to other vegetables, their regular daily intake makes them important targets in carotenoid fortification programs (Wurtzel *et al.* 2012; Zhu *et al.* 2013).

Lutein is the main carotenoid present in the endosperm of wheat (Hentschel *et al.* 2002; Panfili *et al.* 2004; Digesù *et al.* 2009). The first step in carotenoid biosynthesis, which is considered to be a rate-limiting step, is regulated by phytoene synthase (PSY). This enzyme condenses two molecules of geranyl geranyl pyrophosphate (GGPP) to produce a molecule of phytoene (Hirschberg 2001). PSY is encoded by three paralogous genes, *Psy1*, *Psy2* and *Psy3* (Li *et al.* 2008). The role of *Psy1* in the synthesis of carotenoids has been well documented. Indeed, since the first mapping of this gene in chromosomes 7A and 7B in durum wheat (Atienza *et al.* 2007a; Pozniak *et al.* 2007), multiple works have demonstrated the existence of allelic variants associated with differences in endosperm color in wheat (reviewed by Ficcó *et al.* 2014). *Psy1* is located on 7H^{ch}S in *Hordeum chilense* Roem. et Schultz. which explains the increase of YPC in the chromosome addition lines of 7H^{ch}S and 7H^{ch} (Alvarez *et al.* 1998). As observed in wheat, *Psy1* has a relevant role for the production of carotenoids in tritordeum grain (Rodríguez-Suárez *et al.* 2014). Tritordeum is the amphiploid resulting from the cross between wild barley *H. chilense* and durum wheat (Martin and Sánchez-Monge 1982). Preliminary studies showed that tritordeum had a higher YPC than durum wheat (Alvarez *et al.* 1999; Ballesteros *et al.* 2005), while later works aiming to characterize its individual carotenoid composition have reported a higher pigment content in tritordeum. As observed in wheat, lutein is the main carotenoid present in tritordeum but lutein concentration of tritordeum is 5-8 times higher than that in durum wheat.. In addition, a significant proportion of lutein is esterified with fatty acids in tritordeum whereas durum wheat shows very low contents of these esters (Atienza *et al.* 2007b; Mellado-Ortega and Hornero-Méndez 2012; Ahmad *et al.* 2013). Moreover, lutein displays a distinctive profile of esterification with palmitic and linoleic acids in tritordeum (Mellado-Ortega and Hornero-Méndez 2012) which is caused by *H. chilense* genome as recently demonstrated (Mellado-Ortega and Hornero-Méndez 2015) although the genetics of xanthophyll esterification in plants, and in particular lutein esterification in wheat and related species, is poorly understood.

Although only *Psy1* has been related to endosperm carotenoid content in grasses (Li *et al.* 2008; Li *et al.* 2009), *Psy2* may have some importance in tritordeum (Rodríguez-Suárez *et al.* 2014). Both *Psy2* and *Psy3* are located on chromosome 5H^{ch} (Rodríguez-Suárez and Atienza 2012) and thus their potential contribution cannot be neglected.

The development of wheat-alien translocations has allowed the improvement of carotenoid content in both durum [reviewed by Ceoloni *et al.* (2014)] and common wheat (Zhang *et al.* 2005). Chromosome substitution lines are useful for evaluating the substitution effect of wheat by alien chromosomes [reviewed by Khlestkina (2014)]. Thus, translocation lines or chromosome substitution lines for 7H^{ch} and 5H^{ch} would allow the determination of the substitution effect of wheat by *H. chilense* genes for carotenoid content in grain.

At the same time, these materials would allow one to investigate whether the enhancement of endosperm carotenoid content may affect the profile and concentration of lutein esters. Reports on the synthesis of lutein esters in wheat and related cereals are scant and mainly limited to changes during seed storage (Kaneko *et al.* 1995; Kaneko and Oyanagi 1995). Previous studies in tritordeum and *H. chilense* have produced strong evidence for xanthophylls acyltransferase enzymes in *H. chilense* genome (Mellado-Ortega and Hornero-Méndez 2012; Mellado-Ortega and Hornero-Méndez 2015), but the possibility of lutein esters being a direct function of total lutein content remains an open question. Indeed, the increase in lutein esters during storage in a high lutein common wheat line developed at The Waite Campus, University of Adelaide (Ahmad *et al.* 2013), and in tritordeum (Mellado-Ortega and Hornero-Méndez 2015) could back up this hypothesis.

This work aims to determine whether the introgression of chromosomes 5H^{ch} and 7H^{ch} into a wheat background affects its total carotenoid content and lutein esterification profile. A second objective proposed to investigate whether these changes were dependent on the wheat homoeologous chromosome substituted.

Materials and methods

Plant material and experimental design

Thirteen wheat genetic stocks with introgressions from *H. chilense* were used to study the effect of *Psy1* from *H. chilense* in the carotenoid content of endosperm (Table 1). This set of lines comprised four genetic stocks recently described (Mattera *et al.* 2015) including two translocation lines (T7H^{ch}S·5AL

and T7H^{ch}S·2DS), one disomic substitution [DS 7H^{ch}(7D)] and one ditelosomic addition (Dt 7H^{ch}L). A series of common wheat-*H. chilense* substitution lines developed at John Innes Centre (Norwich) corresponding to chromosomes 5H^{ch} and 7H^{ch} were also included (Table 1) (Miller & Reader, unpublished results) (https://www.jic.ac.uk/germplasm/Wheat-Precise-Genetic_stocks-Aliens.pdf). All these genetic stocks were developed in the background of common wheat 'Chinese Spring'. Durum wheat 'Kofa' and tritordeum 'HT621' (Ballesteros *et al.* 2005) were also included as controls. Seedlings were first grown in a glasshouse under semi-controlled conditions and then transplanted to field conditions following a completely randomized block design with 5 replications. For carotenoid analysis, samples at harvest stage (mature grains) from each block were used. Genomic DNA was extracted using the CTAB method according to (Murray and Thompson 1980) from genetic stocks. The presence of *Psy1* from *H. chilense* was assessed using the CAP marker developed by (Atienza *et al.* 2007a) which differentiates between *H. chilense* and wheat homoeologues *Psy1* genes

Extraction of carotenoids

Total carotenoid was extracted from grains using the method described by (Atienza *et al.* 2007b) with some modifications. Two grams of grain sample with 5 mL of HPLC grade acetone (containing 0.1% BHT) were milled in an oscillating ball mill Retsch Model MM400 (Retsch, Haan, Germany) with two stainless-steel ball (1 cm Ø) at 25 Hz for 1 min. All samples were milled in duplicate and a known amount of internal standard (canthaxanthin) was added at the beginning. The resulting slurry was placed in a centrifuge tube (15 mL) and centrifuged at 4,500×g for 5 min at 4 °C. The acetone phase was transferred to another plastic centrifuge tube and the solvent was evaporated under nitrogen stream. The concentrated pigment was dissolved in 1 mL of HPLC grade acetone and stored at -30 °C until chromatographic analysis (HPLC). To prevent photo-degradation of carotenoids, the whole process was carried out under dimmed light. Prior to chromatographic analysis, all the samples were centrifuged at 13,000×g.

Pigment Identification

The procedures for the isolation and identification of carotenoid pigments and their esters have already been described in previous works (Atienza *et al.* 2007b; Mellado-Ortega and Hornero-Méndez 2012).

HPLC analysis of carotenoids

Quantitative HPLC analysis of carotenoids was carried out according to the method of (Mínguez-Mosquera and Hornero-Méndez 1993) with some modifications (Atienza *et al.* 2007b). The HPLC system consisted of a Waters 2695 Alliance chromatograph fitted with a Waters 2998 photodiode array detector, and controlled with Empower2 software (Waters Cromatografía, S.A., Barcelona, Spain). A reversed-phase column (Mediterranea SEA18, 3 μ m, 20×0.46 cm; Teknokroma, Barcelona, Spain) was used. Separation was achieved by a binary-gradient elution using an initial composition of 75% acetone and 25% deionized water, which was increased linearly to 95% acetone in 10 min, then raised to 100% in 2 min, and maintained constant for 10 min. Initial conditions were reached in 5 min. An injection volume of 10 μ L and a flow rate of 1 mL/min were used. Detection was performed at 450 nm, and the online spectra were acquired in the 350-700 nm wavelength range. Quantification was carried out using calibration curves prepared with lutein, α - and β -carotene and zeaxanthin standards isolated and purified from natural sources (Mínguez-Mosquera and Hornero-Méndez 1993). Calibration curves were prepared in the pigment concentration range of 0.5-45 μ g/ml. Lutein esters contents were estimated by using the calibration curve for free lutein, since the esterification of xanthophylls with fatty acids does not modify the chromophore properties. The calibration curve of free lutein was also used to determine the concentration of the *cis*-isomers of lutein. Data were expressed as μ g/g fresh weight.

Statistical analyses

Differences in total carotenoids in mature grains were established using Tukey's Honestly Significant Difference (HSD) test at ($p < 0.05$) after analysis of variance using Statistix v. 9.0. Samples with improved carotenoid content with respect to the common wheat control 'Chinese Spring' were subjected to further analyses for the following parameters: total free lutein, lutein monoesters (lutein-ME), lutein diesters (lutein-DE), trans- β -carotene, trans-zeaxanthin, lutein monoesters (lutein monolinoleate and lutein monopalmitate), lutein diesters (lutein dilinoleate, lutein linoleate-palmitate and lutein dipalmitate).

Results

The genetic stocks used in this work (Table 1) were checked for the presence of *Psy1* from *H. chilense* using a diagnostic CAP marker which differentiates between *H. chilense* and wheat homoeologues *Psy1* genes (Atienza et al. 2007a) despite *Psy1* sequences from *H. chilense* showing a high similarity to those of wheat [similarity of 96.5% with A genome, 95.8% with B genome and 97.5% with D genome (Rodríguez-Suárez et al. 2011)]. As expected, all the genotypes carrying the 7H^{ch}S chromosome arm had this gene while it was absent in the genotypes carrying 5H^{ch} or 7H^{ch}L. Carotenoid profiles of all the genetic stocks were determined in mature grains. The pigment profile consisted of lutein (mainly the all-*trans* isomer together with small amounts of 9-*cis* and 13-*cis* isomers), all-*trans*-zeaxanthin and β-carotene (Fig. 1). A variable proportion of lutein was esterified with fatty acids, either as lutein monoester or lutein diester (referred to hereinafter as lutein-ME and lutein-DE, respectively).

All the genotypes carrying *Psy1* from *H. chilense* had a higher grain carotenoid content than 'Chinese Spring' (Fig. 2). This comprised the four disomic substitution lines [DS 7H^{ch}(7A), DS 7H^{ch}(7B), and two DS 7H^{ch}(7D) lines from different origins]; two ditelosomic substitution lines DS 7H^{ch}S(7A) and DS 7H^{ch}S(7D); and two translocation lines T7H^{ch}S-5AL and T7H^{ch}S-2DS. The majority of these genotypes reached the carotenoid content of durum wheat 'Kofa' (Fig. 2). The only exceptions were the ditelosomic substitution lines DS 7H^{ch}S(7A) and DS 7H^{ch}S(7D). On the contrary, the chromosome substitution lines carrying *Psy2* and *Psy3* [DS 5H^{ch}(5A), DS 5H^{ch}(5B) and DS 5H^{ch}(5D)] did not differ from the wheat control 'Chinese Spring' for total carotenoid content.

Further statistical analyses were performed considering only the genetic stocks with improved carotenoid content relative to common wheat. Significant differences were detected between genetic stocks in which the same wheat chromosome had been substituted. Indeed, both ditelosomic substitution lines DS 7H^{ch}S(7A) and DS 7H^{ch}S(7D) had lower carotenoid contents than their respective disomic substitution lines DS 7H^{ch}(7A) and DS 7H^{ch}(7D) (Fig. 2). This suggests an important role of the arm 7H^{ch}L in the seed carotenoid content. However, this role is only evident when 7H^{ch}S is also present since the addition of 7H^{ch}L did not increase the carotenoid content as evidenced in Dt7H^{ch}L (Fig. 2).

Lutein accounted for around 90% of the total carotenoid content in all the samples carrying *Psy1* (Table 1). However, differences in the contents of free lutein, lutein-ME and lutein-DE were detected (Fig. 3A). This is especially relevant since data on lutein esterification are scant. All the genotypes, except DS 7H^{ch}S(7D), had a higher lutein-ME content than 'Chinese Spring'. The best genotype was DS 7H^{ch}(7A) with 0.59 µg/g of lutein-ME while the tritordeum line HT621 showed 0.63 µg/g. Surprisingly, differences between homoeologous substitutions were found. Indeed, both DS 7H^{ch}(7A) and DS 7H^{ch}(7B) exhibited a higher lutein-ME content than DS 7H^{ch}(7D) which indicates an important role of chromosome 7D in lutein esterification. Similarly, the ditelosomic substitution DS 7H^{ch}S(7A) had a lower lutein-ME content than the complete chromosome substitutions DS 7H^{ch}(7A), which also suggests an important role of 7H^{ch} chromosome in lutein esterification. The lutein-DE content of DS 7H^{ch}(7A) and DS 7H^{ch}(7B) was 0.43 and 0.41 µg/g, respectively. This represented an 11-fold increment relative to 'Chinese Spring' (0.04 µg/g) and more than an 8-fold content with respect to tritordeum 'HT621' (0.05 µg/g).

The contribution of lutein-ME and lutein-DE relative to the lutein pool were also calculated (Fig. 3B). The absence of 7D results in low proportion of lutein-DE (less than 1.0%) independently of the total lutein content. It is worth mentioning that the proportion of lutein-DE in 'Chinese Spring' is higher than in genotypes in which the 7D chromosome is absent, despite its lower carotenoid content. This indicates that lutein esterification is not a direct function of lutein content. On the contrary, specific esterification ability is involved and chromosomes 7D and 7H^{ch} have an important role. Indeed, the simultaneous presence of 7H^{ch} and 7D results in higher levels of lutein-DE and a higher relative contribution of lutein-DE to the lutein pool (Fig. 3A and 3B).

Lutein-ME comprised two different compounds, lutein monolinoleate and lutein monopalmitate, in agreement with previous studies (Mellado-Ortega and Hornero-Méndez 2012) (Fig. 4). The majority of the genetic stocks increased their contents of lutein monolinoleate and lutein monopalmitate compared to 'Chinese Spring' (Fig. 4A). The most notable exception was DS 7H^{ch}S(7D), in which no significant levels of lutein-DE were detected. Similarly, T7H^{ch}S·5AL did not differ from CS for lutein monolinoleate (Fig. 4A). The 7H^{ch}(7D) lines showed a higher proportion of lutein monopalmitate as happened in 'HT621' (Fig. 4B). Conversely, all the genetic stocks in which 7D chromosome was present yielded a similar proportion of

both lutein monopalmitate and lutein monolinoleate as occurred in 'Chinese Spring' (Fig. 4). The pool of lutein-DE comprised three different compounds, lutein dilinoleate, lutein dipalmitate and lutein linoleate-palmitate (Fig 5). Only DS 7H^{ch}(7A), DS 7H^{ch}(7B) and DS 7H^{ch}S(7A) had higher levels of these compounds with respect to 'Chinese Spring' (Fig. 5A). The relative proportion of each lutein-DE in all three lines was similar to that of 'Chinese Spring', despite the significant differences in the content. Indeed, the rough proportions were 1:2:1 for lutein dilinoleate, lutein linoleate-palmitate and lutein dipalmitate. On the contrary, lutein dipalmitate represented around 50% of lutein-DE in 'HT621' with just about 5% of lutein dilinoleate. Similar proportions were obtained when 7D was absent (Fig. 5B).

Discussion

Lutein was the main carotenoid found in all the genotypes analyzed. This agrees with previous results in tritordeum (Atienza *et al.* 2007b; Mellado-Ortega and Hornero-Méndez 2012; Rodríguez-Suárez *et al.* 2014; Mellado-Ortega *et al.* 2015), *H. chilense* (Mellado-Ortega and Hornero-Méndez 2015) and other Triticeae species (Abdel-Aal *et al.* 2002; Howitt *et al.* 2009; Ficco *et al.* 2014). All the genetic stocks carrying *Psy1* yielded more carotenoids than the common wheat control. On the contrary, *Psy2* and *Psy3* from *H. chilense* (*Psy1*-paralog genes) did not affect the carotenoid content of wheat grains which is in agreement with previous findings in maize (Palaisa *et al.* 2003; Gallagher *et al.* 2004; Li *et al.* 2008; Li *et al.* 2009).

The potential of wild relatives for the enhancement of carotenoid content in wheat has been clearly evidenced in common wheat (Zhang *et al.* 2005) and durum wheat (Ceoloni *et al.* 2014) by developing short translocations involving the homoeologous group 7 chromosomes. Indeed, the commercial cultivar 'Cincinnato' is based on an alien translocation involving chromosome 7 (Ceoloni *et al.* 2014). Moreover, the high-lutein common wheat line used by Ahmad *et al.* (2013) is derived from a cross with 'Indis', a bread wheat developed in South Africa (Marais 1992) which carries a chromosome segment from *Thynopyrum distichum* containing a gene for increased lutein content. Thus, this line is likely a translocation involving *T. distichum* chromatin.

The potential of chromosome 7H^{ch} for the increase of carotenoid content was previously shown using chromosome addition lines (Alvarez *et al.* 1998). Similarly, the important role of *Psy1* from *H. chilense* has been demonstrated using transcriptomic approaches (Rodríguez-Suárez *et al.* 2014) and functional studies (Rodríguez-Suárez *et al.* 2011). Our current results demonstrate the potential of *Psy1* from *H. chilense* for the enhancement of carotenoid content of grain in wheat using euploid combinations. Although translocation lines involving 7H^{ch}S have recently been obtained (Mattera *et al.* 2015), the length of these introgressions must be reduced before their effective use in wheat breeding.

The arm 7H^{ch}L seems to contribute to the seed carotenoid content but its role is only evident when 7H^{ch}S is also present. The existence of two different QTLs for YPC on 7AL (Zhang and Dubcovsky 2008; Blanco *et al.* 2011; Colasuonno *et al.* 2014) might explain the contribution of 7H^{ch}L. Indeed, the distal part of 7H^{ch}L is associated with variations in YPC in *H. chilense* (Rodríguez-Suárez and Atienza 2012). However, the potential of this region for the improvement of carotenoid content in wheat is poor since it requires the simultaneous presence of 7H^{ch}S.

Lutein esterification

Previous works have shown that tritordeum has a higher degree of lutein esterification than durum wheat (Atienza *et al.* 2007b; Mellado-Ortega and Hornero-Méndez 2012), in which no significant amount of lutein esters has been reported. One of the hypotheses that we considered was that the contents of lutein esters could be related to the content of lutein. However, our current results show that the total content of lutein is not a crucial determinant for esterification. Indeed, both DS 7H^{ch}(7A) and DS 7H^{ch}(7B) have 8-fold lutein-DE with respect to 'HT621' despite their lower carotenoid content.

The high carotenoid content and degree of lutein esterification of tritordeum has been proposed as a trait derived from the *H. chilense* genome in our previous works. Recently, the carotenoid profiling of *H. chilense* grains has demonstrated the presence of lutein esters in this species (Mellado-Ortega and Hornero-Méndez 2015) while these compounds are almost absent in durum wheat (Atienza *et al.* 2007b; Ahmad *et al.* 2013; Rodríguez-Suárez *et al.* 2014) in agreement with our current results in 'Kofa'. This

demonstrates that lutein esters in tritordeum are derived from the *H. chilense* genome. Lutein esterification has received little attention in wheat so far. A QTL for lutein esterification was reported in chromosome 2B of common wheat (Howitt *et al.* 2009). Further, the formation of lutein esters during storage has been reported in wheat (Kaneko *et al.* 1995; Kaneko and Oyanagi 1995; Ahmad *et al.* 2013) and tritordeum (Mellado-Ortega *et al.* 2015) but genetics of carotenoid esterification in wheat and related species are still poorly understood.

Our current results indicate an important role of chromosomes 7H^{ch} and 7D in lutein esterification. Indeed, the simultaneous occurrence of both chromosomes results in high levels of lutein esters. Furthermore, the coordinated action of both chromosomes resulted in surprisingly high levels of lutein-DE which exceeded more than 8-fold the values obtained in tritordeum. This exceptional esterification ability resulted in near 50% of esterified lutein in the genotypes with both chromosomes, while lutein esters only accounted for 16% of total lutein in tritordeum. Recently, similar values of lutein esterification have been reported in *H. chilense* (Mellado-Ortega and Hornero-Méndez 2015). Indeed, lutein esters accounted for more than 50% of total lutein content of *H. chilense* PI 531781 accession but these seeds had been subjected to long-term storage since they were obtained from a germplasm bank. Lutein esters increase during storage (Kaneko *et al.* 1995; Kaneko and Oyanagi 1995; Ahmad *et al.* 2013; Mellado-Ortega *et al.* 2015) and thus the relative proportion of lutein esters in PI 531781 cannot be directly compared to our results, where samples were processed shortly after harvesting.

Our results indicate the existence of genes involved in the esterification of xanthophylls, including the key xanthophyll acyltransferase enzymes, in both 7H^{ch} and 7D. Lippold *et al.* (2012) isolated two genes (*PES1* and *PES2*) involved in fatty acid phytol ester synthesis in *Arabidopsis*. Thus, we looked for similar sequences in 7DS and 7DL databases using *PES1* (At1g54570) and *PES2* (At3g26840) sequences as queries in BLASTn analyses at <https://urgi.versailles.inra.fr/blast/blast.php>. *PES1* produced significant matches with two contigs (7DS-3857988 and 7DS-2030320). Then, the sequences of these contigs were used as queries for BLASTx at NCBI web page and they significantly matched the accession EMT31342.1 corresponding to an acyltransferase-like protein from *Aegilops tauschii*. Thus, this gene may be a good candidate for further experimentation in the future in both 7H^{ch} and D chromosomes.

Moreover, the genes in 7D and 7H^{ch} seem to have complementary activities. Indeed, the presence of 7H^{ch} promotes the esterification of lutein with palmitic acid in agreement with previous works in tritordeum (Mellado-Ortega and Hornero-Méndez 2012) and *H. chilense* (Mellado-Ortega and Hornero-Méndez 2015). On the contrary, the simultaneous presence of 7D and 7H^{ch} results in 1:2:1 proportions for lutein dilinoleate: lutein linoleate-palmitate: lutein dipalmitate. Considering that 7H^{ch} has a greater affinity towards palmitic acid, our results suggest either that 7D shows a greater affinity for linoleic acid or that it is indifferent with regard to the fatty acid used for lutein esterification. Also, the presence of both 7H^{ch} and 7D results in much higher levels of lutein-DE which reinforces the idea of complementary functions to overcome a rate-limiting step.

The lower content of lutein-DE in DS 7H^{ch}S(7A) with respect to DS 7H^{ch}(7A) suggests a role of both 7H^{ch}S and 7H^{ch}L in lutein esterification. Firstly, the lack of 7H^{ch}L in DS 7H^{ch}S(7A) does not allow the development of lutein-DE to the levels shown by DS 7H^{ch}(7A). Secondly, all DS 7H^{ch}S(7A), T7H^{ch}S·5AL and T7H^{ch}S·2DS carry 7D and 7H^{ch}S but they differ in the content of lutein-DE. However, we cannot be assured that the length of 7H^{ch}S arm is identical in all three lines. Indeed, both translocations were obtained using the gametocidal chromosome from *Aegilops cylindrica* (Mattera *et al.* 2015), and, thus, it is likely that they have a different length of 7H^{ch}S. If this were true, the higher content of lutein-DE of DS 7H^{ch}S(7A) could be explained by an additional gene for lutein esterification in 7H^{ch}S. Nevertheless, a potential role of 2DL and 5AS arms in lutein esterification cannot be excluded but it does not seem to be likely.

Conclusions

Our results demonstrate that *Psy1* from *H. chilense* is an interesting source for the enhancement of carotenoid content in grains of wheat. Furthermore, both 7H^{ch} and 7D carry key genes for lutein esterification and they show complementary activities for the synthesis of lutein-DE. Indeed, whereas 7H^{ch} preferentially esterifies lutein with palmitic acid, 7D is either indifferent to the fatty acid or it prefers linoleic acid for lutein esterification. Also, 7H^{ch} seems to carry more than one gene for lutein esterification but

further studies should be performed to confirm this hypothesis. The involvement and complementarity of 7H^{ch} and 7D are highly significant considering the scarcity of previous results on lutein esterification in wheat.

Acknowledgements

M.G. Mattera was recipient of a fellowship from Ministerio de Economía y Competitividad (BES-2012-055961). This research was funded by Grant AGL2011-24399, from Ministerio de Economía y Competitividad including FEDER funding. We are grateful to Ana Pozo for her technical assistance. D.H.-M. is a member of the IBERCAROT Network, funded by CYTED (ref. 112RT0445).

References

- Abdel-Aal, ESM, Young, JC, Wood, PJ, Rabalski, I, Hucl, P, Falk, D, Fregeau-Reid, J (2002) Einkorn: A potential candidate for developing high lutein wheat. *Cereal Chemistry* **79**, 455-457.
- Ahmad, FT, Asenstorfer, RE, Soriano, IR, Mares, DJ (2013) Effect of temperature on lutein esterification and lutein stability in wheat grain. *Journal of Cereal Science* **58**, 408-413.
- Alvarez, JB, Martin, LM, Martin, A (1999) Genetic variation for carotenoid pigment content in the amphiploid *Hordeum chilense* × *Triticum turgidum* conv. *durum*. *Plant Breeding* **118**, 187-189.
- Alvarez, JB, Martin, LM, Martín, A (1998) Chromosomal localization of genes for carotenoid pigments using addition lines of *Hordeum chilense* in wheat. *Plant Breeding* **117**, 287-289.
- Atienza, SG, Avila, CM, Martin, A (2007a) The development of a PCR-based marker for *Psy1* from *Hordeum chilense*, a candidate gene for carotenoid content accumulation in tritordeum seeds. *Australian Journal of Agricultural Research* **58**, 767-773.
- Atienza, SG, Ballesteros, J, Martin, A, Hornero-Mendez, D (2007b) Genetic variability of carotenoid concentration and degree of esterification among tritordeum (×*Tritordeum* Ascherson et Graebner) and durum wheat accessions. *Journal of Agricultural and Food Chemistry* **55**, 4244-4251.
- Ballesteros, J, Ramirez, MC, Martinez, C, Atienza, SG, Martin, A (2005) Registration of HT621, a high carotenoid content tritordeum germplasm line. *Crop Sci* **45**, 2662-2663.

- Blanco, A, Colasuonno, P, Gadaleta, A, Mangini, G, Schiavulli, A, Simeone, R, Digesù, AM, De Vita, P, Mastrangelo, AM, Cattivelli, L (2011) Quantitative trait loci for yellow pigment concentration and individual carotenoid compounds in durum wheat. *Journal of Cereal Science* **54**, 255-264.
- Britton, G, Hornero-Mendez, D (1997) Carotenoids and colour in fruit and vegetables. In 'Phytochemistry of fruit and vegetables.' (Eds FA Tomás-Barberán, RJ Robins.) pp. 11-27. Clarendon Press, Oxford (United Kingdom))
- Britton, G, Liaaen-Jensen, S, Pfander, H (2009) 'Carotenoids. Volume 5: Nutrition and Health.' (Basel: Birkhäuser Verlag:
- Ceoloni, C, Kuzmanović, L, Forte, P, Gennaro, A, Bitti, A (2014) Targeted exploitation of gene pools of alien Triticeae species for sustainable and multi-faceted improvement of the durum wheat crop. *Crop and Pasture Science* **65**, 96-111.
- Colasuonno, P, Gadaleta, A, Giancaspro, A, Nigro, D, Giove, S, Incerti, O, Mangini, G, Signorile, A, Simeone, R, Blanco, A (2014) Development of a high-density SNP-based linkage map and detection of yellow pigment content QTLs in durum wheat. *Molecular Breeding* 1-16.
- Cuttriss, AJ, Cazzonelli, CI, Wurtzel, ET, Pogson, BJ (2011) Carotenoids. *Advances in Botanical Research* **58**, 1-36.
- Digesù, AM, Platani, C, Cattivelli, L, Mangini, G, Blanco, A (2009) Genetic variability in yellow pigment components in cultivated and wild tetraploid wheats. *Journal of Cereal Science* **50**, 210-218.
- Fernández-García, E, Carvajal-Lérida, I, Jarén-Galán, M, Garrido-Fernández, J, Pérez-Gálvez, A, Hornero-Méndez, D (2012) Carotenoids bioavailability from foods: From plant pigments to efficient biological activities. *Food Research International* **46**, 438-450.
- Ficco, DBM, Mastrangelo, AM, Trono, D, Borrelli, GM, De Vita, P, Fares, C, Beleggia, R, Platani, C, Papa, R (2014) The colours of durum wheat: a review. *Crop and Pasture Science* **65**, 1-15.
- Gallagher, CE, Matthews, PD, Li, F, Wurtzel, ET (2004) Gene duplication in the carotenoid biosynthetic pathway preceded evolution of the grasses. *Plant Physiology* **135**, 1776-1783.
- Hentschel, V, Kranl, K, Hollmann, J, Lindhauer, MG, Bohm, V, Bitsch, R (2002) Spectrophotometric determination of yellow pigment content and evaluation of carotenoids by high-performance liquid chromatography in durum wheat grain. *Journal of Agricultural and Food Chemistry* **50**, 6663-6668.
- Hirschberg, J (2001) Carotenoid biosynthesis in flowering plants. *Curr. Opin. Plant Biol.* **4**, 210-218.
- Howitt, CA, Cavanagh, CR, Bowerman, AF, Cazzonelli, C, Rampling, L, Mimica, JL, Pogson, BJ (2009) Alternative splicing, activation of cryptic exons and amino acid substitutions in carotenoid biosynthetic genes are associated with lutein accumulation in wheat endosperm. *Functional & Integrative Genomics* **9**, 363-376.
- Kaneko, S, Nagamine, T, Yamada, T (1995) Esterification of endosperm lutein with fatty acids during the storage of wheat seeds. *Bioscience, Biotechnology and Biochemistry* **59**, 1-4.

- Kaneko, S, Oyanagi, A (1995) Varietal differences in the rate of esterification of endosperm lutein during the storage of wheat seeds. *Biosci. Biotech. biochem.* **59**, 2312-2313.
- Khlestkina, E (2014) Current applications of wheat and wheat–alien precise genetic stocks. *Molecular Breeding* **34**, 273-281.
- Landrum, JT, Bone, RA (2004) Dietary lutein and zeaxanthin: reducing the risk for macular degeneration. *Agro Food Industry Hi-Tech* **15**, 22-25.
- Li, F, Tzfadia, O, Wurtzel, ET (2009) The phytoene synthase gene family in the grasses: Subfunctionalization provides tissue-specific control of carotenogenesis. *Plant Signaling and Behavior* **4**, 208-211.
- Li, FQ, Vallabhaneni, R, Yu, J, Rocheford, T, Wurtzel, ET (2008) The maize phytoene synthase gene family: Overlapping roles for carotenogenesis in endosperm, photomorphogenesis, and thermal stress tolerance. *Plant Physiology* **147**, 1334-1346.
- Lippold, F, Dorp, K, Abraham, M, Hölzl, G, Wewer, V, Yilmaz, JL, Lager, I, Montandon, C, Besagni, C, Kessler, F, Stymne, S, Dörmann, P (2012) Fatty acid phytol ester synthesis in chloroplasts of *Arabidopsis*. *Plant Cell* **24**, 2001-2014
- Marais, GF (1992) The modification of a common wheat-*Thinopyrum distichum* translocated chromosome with a locus homeoallelic to Lr19. *Theoretical and Applied Genetics* **85**, 73-78.
- Mares, DJ, Campbell, AW (2001) Mapping components of flour colour in Australian wheat. *Australian Journal of Agricultural Research* **52**, 1297-1309.
- Martin, A, Sánchez-Monge, E (1982) Cytology and morphology of the amphiploid *Hordeum chilense* × *Triticum turgidum* conv. *durum*. *Euphytica* **31**, 261-267.
- Mattera, MG, Avila, CM, Atienza, SG, Cabrera, A (2015) Cytological and molecular characterization of wheat-*Hordeum chilense* chromosome 7H^{ch} introgression lines. *Euphytica* **203**, 165-176.
- Mellado-Ortega, E, Atienza, SG, Hornero-Méndez, D (2015) Carotenoid evolution during postharvest storage of durum wheat (*Triticum turgidum* conv. *durum*) and tritordeum (×*Tritordeum* Ascherson et Graebner) grains. *Journal of Cereal Science* **62**, 134-142.
- Mellado-Ortega, E, Hornero-Méndez, D (2012) Isolation and identification of lutein esters, including their regioisomers, in tritordeum (×*Tritordeum* Ascherson et Graebner) grains: Evidence for a preferential xanthophyll acyltransferase activity. *Food Chemistry* **135**, 1344-1352.
- Mellado-Ortega, E, Hornero-Méndez, D (2015) Carotenoid profiling of *Hordeum chilense* grains: The parental proof for the origin of the high carotenoid content and esterification pattern of tritordeum. *Journal of Cereal Science* **62**, 15-21.
- Mínguez-Mosquera, MI, Hornero-Méndez, D (1993) Separation and quantification of the carotenoid pigments in red peppers (*Capsicum annuum* L.), paprika and oleoresin by reversed-phase HPLC. *Journal of Agricultural and Food Chemistry* **41**, 1616-1620.
- Murray, YHG, Thompson, WF (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research* **8**, 4321-4326.

- Palaisa, KA, Morgante, M, Williams, M, Rafalski, A (2003) Contrasting effects of selection on sequence diversity and linkage disequilibrium at two phytoene synthase loci. *Plant Cell* **15**, 1795-1806.
- Panfili, G, Fratianni, A, Irano, M (2004) Improved normal-phase high-performance liquid chromatography procedure for the determination of carotenoids in cereals. *Journal of Agricultural and Food Chemistry* **52**, 6373-6377.
- Pozniak, CJ, Knox, RE, Clarke, FR, Clarke, JM (2007) Identification of QTL and association of a phytoene synthase gene with endosperm colour in durum wheat. *Theoretical and Applied Genetics* **114**, 525-537.
- Rodríguez-Suárez, C, Atienza, SG (2012) *Hordeum chilense* genome, a useful tool to investigate the endosperm yellow pigment content in the Triticeae. *BMC Plant Biology* **12**, 200.
- Rodríguez-Suárez, C, Atienza, SG, Pistón, F (2011) Allelic variation, alternative splicing and expression analysis of *Psy1* gene in *Hordeum chilense* Roem. et Schult. *PLoS ONE* **6**, e19885.
- Rodríguez-Suárez, C, Giménez, MJ, Atienza, SG (2010) Progress and perspectives for carotenoid accumulation in selected Triticeae species. *Crop and Pasture Science* **61**, 743-751.
- Rodríguez-Suárez, C, Mellado-Ortega, E, Hornero-Méndez, D, Atienza, SG (2014) Increase in transcript accumulation of *Psy1* and *e-Lcy* genes in grain development is associated with differences in seed carotenoid content between durum wheat and tritordeum. *Plant Molecular Biology* **84**, 659-673.
- WHO (2009) Global prevalence of vitamin A deficiency in populations at risk 1995-2005. *WHO Global database on Vitamin A deficiency*
- Wurtzel, ET, Cuttriss, A, Vallabhaneni, R (2012) Maize provitamin A carotenoids, current resources and future metabolic engineering challenges. *Frontiers in Plant Science* **3**,
- Zhang, W, Dubcovsky, J (2008) Association between allelic variation at the *Phytoene synthase 1* gene and yellow pigment content in the wheat grain. *Theoretical and Applied Genetics* **116**, 635-645.
- Zhang, WJ, Lukaszewski, AJ, Kolmer, J, Soria, MA, Goyal, S, Dubcovsky, J (2005) Molecular characterization of durum and common wheat recombinant lines carrying leaf rust resistance (*Lr19*) and yellow pigment (Y) genes from *Lophopyrum ponticum*. *Theoretical and Applied Genetics* **111**, 573-582.
- Zhu, C, Sanahuja, G, Yuan, D, Farré, G, Arjó, G, Berman, J, Zorrilla-López, U, Banakar, R, Bai, C, Pérez-Massot, E, Bassie, L, Capell, T, Christou, P (2013) Biofortification of plants with altered antioxidant content and composition: genetic engineering strategies. *Plant Biotechnology Journal* **11**, 129-141.

Table 1. Plant material used in this work

DS, Disomic substitution; T, Translocation; WGS, wheat genetic stocks.

Lines 1-4 were described by Mattera et al. (2015) and lines 5-13 were

obtained by T.E. Miller and S.M. Reader:

www.jic.ac.uk/germplasm/Wheat-Precise-Genetic_stocks-Aliens.pdf

Line ¹	Species	Substituted chromosome
DS 7H ^{ch} (7D)*	WGS	7D
T7H ^{ch} S-5AL*	WGS	5AS
T7H ^{ch} S-2DS*	WGS	2DL
Dt 7H ^{ch} L*	WGS	None
DS 5H ^{ch} (5A)	WGS	5A
DS 5H ^{ch} (5B)	WGS	5B
DS 5H ^{ch} (5D)	WGS	5D
DS 7H ^{ch} (7A)	WGS	7A
DS 7H ^{ch} S (7A)	WGS	7A
DS 7H ^{ch} (7B)	WGS	7B
DS 7H ^{ch} L (7B)	WGS	7B
DS 7H ^{ch} S (7D)	WGS	7D
DS 7H ^{ch} (7D)	WGS	7D
'HT621'	× <i>Tritordeum</i>	
'Kofa'	<i>T. turgidum</i> subsp. <i>durum</i>	
'Chinese Spring'	<i>T. aestivum</i>	

Table 2 Carotenoid composition expressed in µg/g dry weight of genetic stocks with improved total carotenoid content

DS, Disomic substitution; T, Translocation. All genetic stocks were developed in 'Chinese Spring' background. Within columns, means followed by the same letter are not significantly different at $P = 0.05$ determined by Tukey's HSD test; n.d., not detected

Genotype	Free lutein ^A	All- <i>trans</i> -zeaxanthin	All- <i>trans</i> -β-carotene	Lutein monoesters ^B	Lutein diesters ^C	Total carotenoids
'Chinese Spring'	0.36 ± 0.02 ^f	0.12 ± 0.01 ^b	0.01 ± 0.00 ^e	0.11 ± 0.00 ^d	0.04 ± 0.00 ^c	0.64±0.03 ^d
DS 7H ^{ch} (7A)	1.07 ± 0.08 ^{cde}	0.14 ± 0.01 ^{ab}	0.09 ± 0.01 ^a	0.59 ± 0.02 ^a	0.43 ± 0.01 ^a	2.33±0.09 ^a
DS 7H ^{ch} (7B)	0.75 ± 0.02 ^e	0.14 ± 0.00 ^{ab}	0.04 ± 0.00 ^d	0.47 ± 0.02 ^b	0.41 ± 0.03 ^a	1.81±0.04 ^b
DS 7H ^{ch} (7D)	1.81 ± 0.09 ^a	0.16 ± 0.01 ^a	0.06 ± 0.00 ^{bc}	0.27 ± 0.02 ^c	0.02 ± 0.00 ^c	2.32±0.10 ^a
DS 7H ^{ch} (7D) ^D	1.52 ± 0.10 ^{ab}	0.15 ± 0.01 ^{ab}	0.06 ± 0.00 ^{bcd}	0.29 ± 0.01 ^c	0.02 ± 0.00 ^c	2.04±0.10 ^{ab}
DS 7H ^{ch} S(7A)	0.92 ± 0.03 ^{de}	0.14 ± 0.01 ^{ab}	0.06 ± 0.00 ^{bcd}	0.41 ± 0.02 ^b	0.13 ± 0.01 ^b	1.66±0.01 ^{bc}
DS 7H ^{ch} S(7D)	1.13 ± 0.13 ^{cd}	0.12 ± 0.02 ^b	0.02 ± 0.00 ^e	0.08 ± 0.01 ^d	n.d.	1.35±0.16 ^c
T7H ^{ch} S-5AL ^D	1.72 ± 0.09 ^a	0.15 ± 0.01 ^{ab}	0.05 ± 0.00 ^{cd}	0.20 ± 0.04 ^c	0.01 ± 0.00 ^c	2.13±0.13 ^{ab}
T7H ^{ch} S-2DS ^D	1.35 ± 0.02 ^{bc}	0.11 ± 0.00 ^b	0.06 ± 0.00 ^b	0.23 ± 0.01 ^c	0.01 ± 0.00 ^c	1.76±0.03 ^{bc}

^AFree lutein = all-*trans*-Lutein + 9-*cis*-Lutein + 13-*cis*-Lutein.

^BLutein monoesters = Lutein monopalmitate + Lutein monolinoleate.

^CLutein diesters = Lutein dilinoleate + Lutein dipalmitate + Lutein linoleate-palmitate.

^DDescribed by Mattera *et al.* (2015).

Caption Figures

Fig 1. HPLC chromatogram corresponding to the carotenoid profile of DS 7Hch (7A). Peak identities: 1, all-*trans*-zeaxanthin; 2, all-*trans*-lutein; 3, 9-*cis*-lutein; 4, 13-*cis*-lutein; 5, lutein monopalmitate; 6, lutein monolinoleate; 7, all-*trans*- β -carotene; 8, lutein dilinoleate; 9 lutein linoleate-palmitate; 10, lutein dipalmitate; Lutein-ME, lutein monoesters; Lutein-DE, lutein diesters; IS, internal standard (all-*trans*-canthaxanthin). Detection wavelength was 450 nm.

Fig 2. Carotenoid content in grain of wheat genetic stocks. Total carotenoids are expressed in $\mu\text{g/g}$ dry weight. Different letters indicate significant differences at $p < 0.05$ determined by Tukey's HSD test.

Fig 3. Lutein profile in wheat genetic stocks with improved carotenoid content relative to ‘Chinese Spring’. A. Contents of free lutein, lutein monoester (lutein-ME) and lutein diester (lutein-DE). B. Relative contribution of each compound (%) to the total content of lutein. Tritordeum ‘HT621’ and durum wheat ‘Kofa’ are shown only as references since they were not included in the ANOVA analysis. For each compound, different letters indicate significant differences at $p < 0.05$ determined by Tukey’s HSD test.

Fig 4. Lutein monoesters (lutein-ME) profile in the genetic stocks with improved carotenoid content. A. Contents of lutein monolinoleate and lutein monopalmitate. B. Relative contribution of each monoester (%) to the total content of lutein-ME. Tritordeum ‘HT621’ is shown only as reference since it was not included in the ANOVA analysis. For each compound, different letters indicate significant differences at $p < 0.05$ determined by Tukey’s HSD test.

Fig 5. Lutein diester (lutein-DE) profile in the genetic stocks with improved carotenoid content relative to ‘Chinese Spring’. A. Grain contents of lutein dilinoleate, lutein linoleate-palmitate and lutein dipalmitate. B. Relative contribution of each diester (%) to the total content of lutein-DE. Tritordeum ‘HT621’ is shown only as reference since it was not included in the ANOVA analysis. For each compound, different letters indicate significant differences at $p < 0.05$ determined by Tukey’s HSD test.