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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.

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

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 ($T7H^{ch}S{\cdot}5AL$

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 ºC. 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 ºC 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).

159 **HPLC analysis of carotenoids**

160 Quantitative HPLC analysis of carotenoids was carried out according to the method of (Mínguez-
161 Mosquera and Hornero-Méndez 1993) with some modifications (Atienza *et al.* 2007b). The HPLC system
162 consisted of a Waters 2695 Alliance chromatograph fitted with a Waters 2998 photodiode array detector,
163 and controlled with Empower2 software (Waters Cromatografía, S.A., Barcelona, Spain). A reversed-
164 phase column (Mediterranea SEA18, 3 µm, 20×0.46 cm; Teknokroma, Barcelona, Spain) was used.
165 Separation was achieved by a binary-gradient elution using an initial composition of 75% acetone and
166 25% deionized water, which was increased linearly to 95% acetone in 10 min, then raised to 100% in 2
167 min, and maintained constant for 10 min. Initial conditions were reached in 5 min. An injection volume of
168 10 µL and a flow rate of 1 mL/min were used. Detection was performed at 450 nm, and the online spectra
169 were acquired in the 350-700 nm wavelength range. Quantification was carried out using calibration
170 curves prepared with lutein, α- and β-carotene and zeaxanthin standards isolated and purified from natural
171 sources (Mínguez-Mosquera and Hornero-Méndez 1993). Calibration curves were prepared in the pigment
172 concentration range of 0.5-45 µg/ml. Lutein esters contents were estimated by using the calibration curve
173 for free lutein, since the esterification of xanthophylls with fatty acids does not modify the chromophore
174 properties. The calibration curve of free lutein was also used to determine the concentration of the *cis*-
175 isomers of lutein. Data were expressed as µg/g fresh weight.

176 **Statistical analyses**

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

183

184 **Results**

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

195 All the genotypes carrying *Psy1* from *H. chilense* had a higher grain carotenoid content than
196 'Chinese Spring' (Fig. 2). This comprised the four disomic substitution lines [DS 7H^{ch}(7A), DS 7H^{ch}(7B),
197 and two DS 7H^{ch}(7D) lines from different origins]; two ditelosomic substitution lines DS 7H^{ch}S(7A) and DS
198 7H^{ch}S(7D); and two translocation lines T7H^{ch}S·5AL and T7H^{ch}S·2DS. The majority of these genotypes
199 reached the carotenoid content of durum wheat 'Kofa' (Fig. 2). The only exceptions were the ditelosomic
200 substitution lines DS 7H^{ch}S(7A) and DS 7H^{ch}S(7D). On the contrary, the chromosome substitution lines
201 carrying *Psy2* and *Psy3* [DS 5H^{ch}(5A), DS 5H^{ch}(5B) and DS 5H^{ch}(5D)] did not differ from the wheat control
202 'Chinese Spring' for total carotenoid content.

203 Further statistical analyses were performed considering only the genetic stocks with improved
204 carotenoid content relative to common wheat. Significant differences were detected between genetic
205 stocks in which the same wheat chromosome had been substituted. Indeed, both ditelosomic substitution
206 lines DS 7H^{ch}S(7A) and DS 7H^{ch}S(7D) had lower carotenoid contents than their respective disomic
207 substitution lines DS 7H^{ch}(7A) and DS 7H^{ch}(7D) (Fig. 2). This suggests an important role of the arm 7H^{ch}L
208 in the seed carotenoid content. However, this role is only evident when 7H^{ch}S is also present since the
209 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

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

245

246 **Discussion**

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

255 The potential of wild relatives for the enhancement of carotenoid content in wheat has been
256 clearly evidenced in common wheat (Zhang *et al.* 2005) and durum wheat (Ceoloni *et al.* 2014) by
257 developing short translocations involving the homoeologous group 7 chromosomes. Indeed, the
258 commercial cultivar 'Cincinnato' is based on an alien translocation involving chromosome 7 (Ceoloni *et al.*
259 2014). Moreover, the high-lutein common wheat line used by Ahmad *et al.* (2013) is derived from a cross
260 with 'Indis', a bread wheat developed in South Africa (Marais 1992) which carries a chromosome segment
261 from *Thynopyrum distichum* containing a gene for increased lutein content. Thus, this line is likely a
262 translocation involving *T. distichum* chromatin.

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

270 The arm 7H^{ch}L seems to contribute to the seed carotenoid content but its role is only evident
271 when 7H^{ch}S is also present. The existence of two different QTLs for YPC on 7AL (Zhang and Dubcovsky
272 2008; Blanco *et al.* 2011; Colasuonno *et al.* 2014) might explain the contribution of 7H^{ch}L. Indeed, the
273 distal part of 7H^{ch}L is associated with variations in YPC in *H. chilense* (Rodríguez-Suárez and Atienza
274 2012). However, the potential of this region for the improvement of carotenoid content in wheat is poor
275 since it requires the simultaneous presence of 7H^{ch}S.

276

277 Lutein esterification

278 Previous works have shown that tritordeum has a higher degree of lutein esterification than
279 durum wheat (Atienza *et al.* 2007b; Mellado-Ortega and Hornero-Méndez 2012), in which no significant
280 amount of lutein esters has been reported. One of the hypotheses that we considered was that the
281 contents of lutein esters could be related to the content of lutein. However, our current results show that
282 the total content of lutein is not a crucial determinant for esterification. Indeed, both DS 7H^{ch}(7A) and DS
283 7H^{ch}(7B) have 8-fold lutein-DE with respect to 'HT621' despite their lower carotenoid content.

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

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

295 Our current results indicate an important role of chromosomes $7H^{ch}$ and 7D in lutein esterification.
296 Indeed, the simultaneous occurrence of both chromosomes results in high levels of lutein esters.
297 Furthermore, the coordinated action of both chromosomes resulted in surprisingly high levels of lutein-DE
298 which exceeded more than 8-fold the values obtained in tritordeum. This exceptional esterification ability
299 resulted in near 50% of esterified lutein in the genotypes with both chromosomes, while lutein esters only
300 accounted for 16% of total lutein in tritordeum. Recently, similar values of lutein esterification have been
301 reported in *H. chilense* (Mellado-Ortega and Hornero-Méndez 2015). Indeed, lutein esters accounted for
302 more than 50% of total lutein content of *H. chilense* PI 531781 accession but these seeds had been
303 subjected to long-term storage since they were obtained from a germplasm bank. Lutein esters increase
304 during storage (Kaneko *et al.* 1995; Kaneko and Oyanagi 1995; Ahmad *et al.* 2013; Mellado-Ortega *et al.*
305 2015) and thus the relative proportion of lutein esters in PI 531781 cannot be directly compared to our
306 results, where samples were processed shortly after harvesting.

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

316 Moreover, the genes in 7D and 7H^{ch} seem to have complementary activities. Indeed, the presence of 7H^{ch}
317 promotes the esterification of lutein with palmitic acid in agreement with previous works in tritordeum
318 (Mellado-Ortega and Hornero-Méndez 2012) and *H. chilense* (Mellado-Ortega and Hornero-Méndez
319 2015). On the contrary, the simultaneous presence of 7D and 7H^{ch} results in 1:2:1 proportions for lutein
320 dilinoleate: lutein linoleate-palmitate: lutein dipalmitate. Considering that 7H^{ch} has a greater affinity towards
321 palmitic acid, our results suggest either that 7D shows a greater affinity for linoleic acid or that it is
322 indifferent with regard to the fatty acid used for lutein esterification. Also, the presence of both 7H^{ch} and 7D
323 results in much higher levels of lutein-DE which reinforces the idea of complementary functions to
324 overcome a rate-limiting step.

325 The lower content of lutein-DE in DS 7H^{ch}S(7A) with respect to DS 7H^{ch}(7A) suggests a role of
326 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
327 development of lutein-DE to the levels shown by DS 7H^{ch}(7A). Secondly, all DS 7H^{ch}S(7A), T7H^{ch}S·5AL
328 and T7H^{ch}S·2DS carry 7D and 7H^{ch}S but they differ in the content of lutein-DE. However, we cannot be
329 assured that the length of 7H^{ch}S arm is identical in all three lines. Indeed, both translocations were
330 obtained using the gametocidal chromosome from *Aegilops cylindrica* (Mattera *et al.* 2015), and, thus, it is
331 likely that they have a different length of 7H^{ch}S. If this were true, the higher content of lutein-DE of DS
332 7H^{ch}S(7A) could be explained by an additional gene for lutein esterification in 7H^{ch}S. Nevertheless, a
333 potential role of 2DL and 5AS arms in lutein esterification cannot be excluded but it does not seem to be
334 likely.

335

336 **Conclusions**

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

342 further studies should be performed to confirm this hypothesis. The involvement and complementarity of
343 $7H^{ch}$ and 7D are highly significant considering the scarcity of previous results on lutein esterification in
344 wheat.

345

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352 **References**

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354 Abdel-Aal, ESM, Young, JC, Wood, PJ, Rabalski, I, Hucl, P, Falk, D, Fregeau-Reid, J (2002) Einkorn: A
355 potential candidate for developing high lutein wheat. *Cereal Chemistry* **79**, 455-457.
356 Ahmad, FT, Asenstorfer, RE, Soriano, IR, Mares, DJ (2013) Effect of temperature on lutein esterification
357 and lutein stability in wheat grain. *Journal of Cereal Science* **58**, 408-413.
358 Alvarez, JB, Martin, LM, Martin, A (1999) Genetic variation for carotenoid pigment content in the
359 amphiploid *Hordeum chilense* × *Triticum turgidum* conv. *durum*. *Plant Breeding* **118**, 187-189.
360 Alvarez, JB, Martin, LM, Martín, A (1998) Chromosomal localization of genes for carotenoid pigments
361 using addition lines of *Hordeum chilense* in wheat. *Plant Breeding* **117**, 287-289.
362 Atienza, SG, Avila, CM, Martin, A (2007a) The development of a PCR-based marker for *Psy1* from
363 *Hordeum chilense*, a candidate gene for carotenoid content accumulation in tritordeum seeds.
364 *Australian Journal of Agricultural Research* **58**, 767-773.
365 Atienza, SG, Ballesteros, J, Martin, A, Hornero-Mendez, D (2007b) Genetic variability of carotenoid
366 concentration and degree of esterification among tritordeum (×*Tritordeum* Ascherson et
367 Graebner) and durum wheat accessions. *Journal of Agricultural and Food Chemistry* **55**, 4244-
368 4251.
369 Ballesteros, J, Ramirez, MC, Martinez, C, Atienza, SG, Martin, A (2005) Registration of HT621, a high
370 carotenoid content tritordeum germplasm line. *Crop Sci* **45**, 2662-2663.

371 Blanco, A, Colasuonno, P, Gadaleta, A, Mangini, G, Schiavulli, A, Simeone, R, Digesù, AM, De Vita, P,
372 Mastrangelo, AM, Cattivelli, L (2011) Quantitative trait loci for yellow pigment concentration and
373 individual carotenoid compounds in durum wheat. *Journal of Cereal Science* **54**, 255-264.
374 Britton, G, Hornero-Mendez, D (1997) Carotenoids and colour in fruit and vegetables. In 'Phytochemistry
375 of fruit and vegetables.' (Eds FA Tomás-Barberán, RJ Robins.) pp. 11-27. Clarendon Press,
376 Oxford (United Kingdom))
377 Britton, G, Liaaen-Jensen, S, Pfander, H (2009) 'Carotenoids. Volume 5: Nutrition and Health.' (Basel:
378 Birkhäuser Verlag:
379 Ceoloni, C, Kuzmanović, L, Forte, P, Gennaro, A, Bitti, A (2014) Targeted exploitation of gene pools of
380 alien Triticeae species for sustainable and multi-faceted improvement of the durum wheat crop.
381 *Crop and Pasture Science* **65**, 96-111.
382 Colasuonno, P, Gadaleta, A, Giancaspro, A, Nigro, D, Giove, S, Incerti, O, Mangini, G, Signorile, A,
383 Simeone, R, Blanco, A (2014) Development of a high-density SNP-based linkage map and
384 detection of yellow pigment content QTLs in durum wheat. *Molecular Breeding* 1-16.
385 Cuttriss, AJ, Cazzonelli, CI, Wurtzel, ET, Pogson, BJ (2011) Carotenoids. *Advances in Botanical Research*
386 **58**, 1-36.
387 Digesù, AM, Platani, C, Cattivelli, L, Mangini, G, Blanco, A (2009) Genetic variability in yellow pigment
388 components in cultivated and wild tetraploid wheats. *Journal of Cereal Science* **50**, 210-218.
389 Fernández-García, E, Carvajal-Lérida, I, Jarén-Galán, M, Garrido-Fernández, J, Pérez-Gálvez, A,
390 Hornero-Méndez, D (2012) Carotenoids bioavailability from foods: From plant pigments to
391 efficient biological activities. *Food Research International* **46**, 438-450.
392 Ficco, DBM, Mastrangelo, AM, Trono, D, Borrelli, GM, De Vita, P, Fares, C, Beleggia, R, Platani, C, Papa,
393 R (2014) The colours of durum wheat: a review. *Crop and Pasture Science* **65**, 1-15.
394 Gallagher, CE, Matthews, PD, Li, F, Wurtzel, ET (2004) Gene duplication in the carotenoid biosynthetic
395 pathway preceded evolution of the grasses. *Plant Physiology* **135**, 1776-1783.
396 Hentschel, V, Kranl, K, Hollmann, J, Lindhauer, MG, Bohm, V, Bitsch, R (2002) Spectrophotometric
397 determination of yellow pigment content and evaluation of carotenoids by high-performance liquid
398 chromatography in durum wheat grain. *Journal of Agricultural and Food Chemistry* **50**, 6663-
399 6668.
400 Hirschberg, J (2001) Carotenoid biosynthesis in flowering plants. *Curr. Opin. Plant Biol.* **4**, 210-218.
401 Howitt, CA, Cavanagh, CR, Bowerman, AF, Cazzonelli, C, Rampling, L, Mimica, JL, Pogson, BJ (2009)
402 Alternative splicing, activation of cryptic exons and amino acid substitutions in carotenoid
403 biosynthetic genes are associated with lutein accumulation in wheat endosperm. *Functional &*
404 *Integrative Genomics* **9**, 363-376.
405 Kaneko, S, Nagamine, T, Yamada, T (1995) Esterification of endosperm lutein with fatty acids during the
406 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 lutien 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) Dietaly lutein and zeaxanthin: reducing the risk for macular degeneartion. *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, Kv, Abraham, M, Hölzl, G, Wewer, V, Yilmaz, JL, Lager, I, Montandon, C, Besagni, C, Kessler, F, Stymne, S, Dörmann, P (2012) Fatty acid phytyl 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 (*xTritordeum* 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.

444 Palaisa, KA, Morgante, M, Williams, M, Rafalski, A (2003) Contrasting effects of selection on sequence
445 diversity and linkage disequilibrium at two phytoene synthase loci. *Plant Cell* **15**, 1795-1806.
446 Panfili, G, Fratianni, A, Irano, M (2004) Improved normal-phase high-performance liquid chromatography
447 procedure for the determination of carotenoids in cereals. *Journal of Agricultural and Food*
448 *Chemistry* **52**, 6373-6377.
449 Pozniak, CJ, Knox, RE, Clarke, FR, Clarke, JM (2007) Identification of QTL and association of a phytoene
450 synthase gene with endosperm colour in durum wheat. *Theoretical and Applied Genetics* **114**,
451 525-537.
452 Rodríguez-Suárez, C, Atienza, SG (2012) *Hordeum chilense* genome, a useful tool to investigate the
453 endosperm yellow pigment content in the Triticeae. *BMC Plant Biology* **12**, 200.
454 Rodríguez-Suárez, C, Atienza, SG, Pistón, F (2011) Allelic variation, alternative splicing and expression
455 analysis of *Psy1* gene in *Hordeum chilense* Roem. et Schult. *PLoS ONE* **6**, e19885.
456 Rodríguez-Suárez, C, Giménez, MJ, Atienza, SG (2010) Progress and perspectives for carotenoid
457 accumulation in selected Triticeae species. *Crop and Pasture Science* **61**, 743-751.
458 Rodríguez-Suárez, C, Mellado-Ortega, E, Hornero-Méndez, D, Atienza, SG (2014) Increase in transcript
459 accumulation of *Psy1* and *e-Lcy* genes in grain development is associated with differences in
460 seed carotenoid content between durum wheat and tritordeum. *Plant Molecular Biology* **84**, 659-
461 673.
462 WHO (2009) Global prevalence of vitamin A deficiency in populations at risk 1995-2005. *WHO Global*
463 *database on Vitamin A deficiency*
464 Wurtzel, ET, Cuttriss, A, Vallabhaneni, R (2012) Maize provitamin A carotenoids, current resources and
465 future metabolic engineering challenges. *Frontiers in Plant Science* **3**,
466 Zhang, W, Dubcovsky, J (2008) Association between allelic variation at the *Phytoene synthase 1* gene
467 and yellow pigment content in the wheat grain. *Theoretical and Applied Genetics* **116**, 635-645.
468 Zhang, WJ, Lukaszewski, AJ, Kolmer, J, Soria, MA, Goyal, S, Dubcovsky, J (2005) Molecular
469 characterization of durum and common wheat recombinant lines carrying leaf rust resistance
470 (*Lr19*) and yellow pigment (*Y*) genes from *Lophopyrum ponticum*. *Theoretical and Applied*
471 *Genetics* **111**, 573-582.
472 Zhu, C, Sanahuja, G, Yuan, D, Farré, G, Arjó, G, Berman, J, Zorrilla-López, U, Banakar, R, Bai, C, Pérez-
473 Massot, E, Bassie, L, Capell, T, Christou, P (2013) Biofortification of plants with altered
474 antioxidant content and composition: genetic engineering strategies. *Plant Biotechnology Journal*
475 **11**, 129-141.

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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'	×*Tritordeum*	
'Kofa'	*T. turgidum* subsp. d*urum*	
'Chinese Spring'	*T. aestivum*	

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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-*trans*-zeaxanthin	All-trans-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}

[A]Free lutein = all-*trans*-Lutein + 9-*cis*-Lutein + 13-*cis*-Lutein.

[B]Lutein monoesters = Lutein monopalmitate + Lutein monolinoleate.

[C]Lutein diesters = Lutein dilinoleate + Lutein dipalmitate + Lutein linoleate-palmitate.

[D]Described 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.

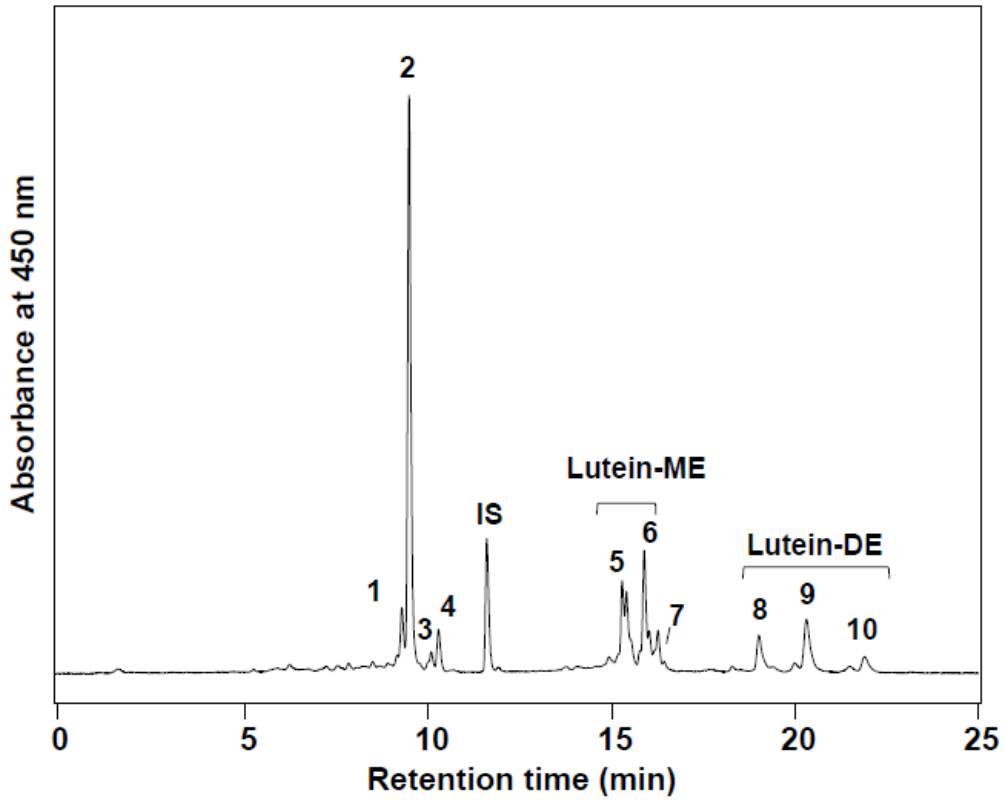

Fig 2. Carotenoid content in grain of wheat genetic stocks. Total carotenoids are expressed in μg/g dry weight. Different letters indicate significant differences at $p < 0.05$ determined by Tukey's HSD test.

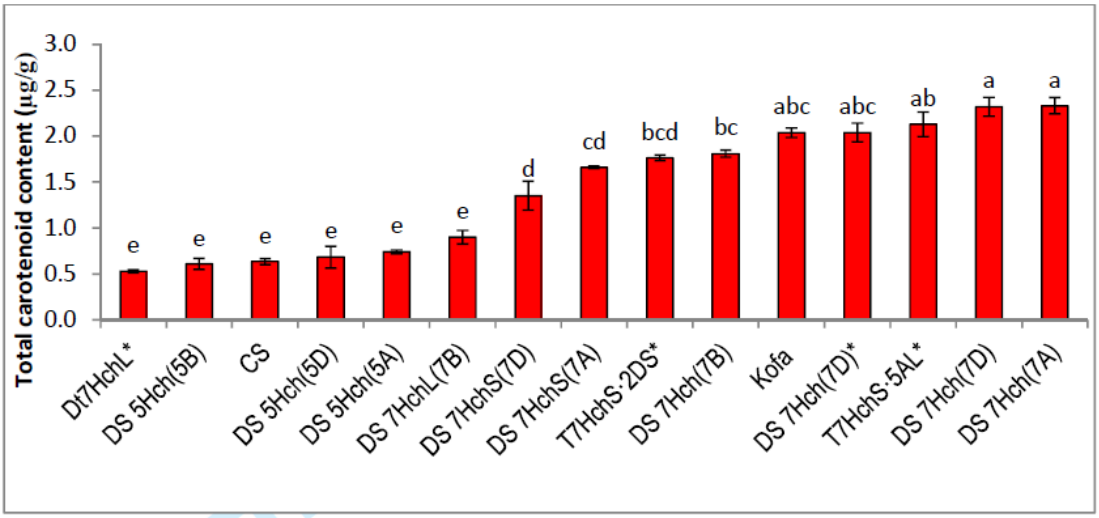

Fig 3. Lutein profile in wheat genetic stocks with improved carotenoid content relative to 'Chinese Spring'. A. Contents of free lutein, lutein monoester (lutein-ME) and lutein diester (lutein-DE). B. Relative contribution of each compound (%) to the total content of lutein. Tritordeum 'HT621' and durum 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.

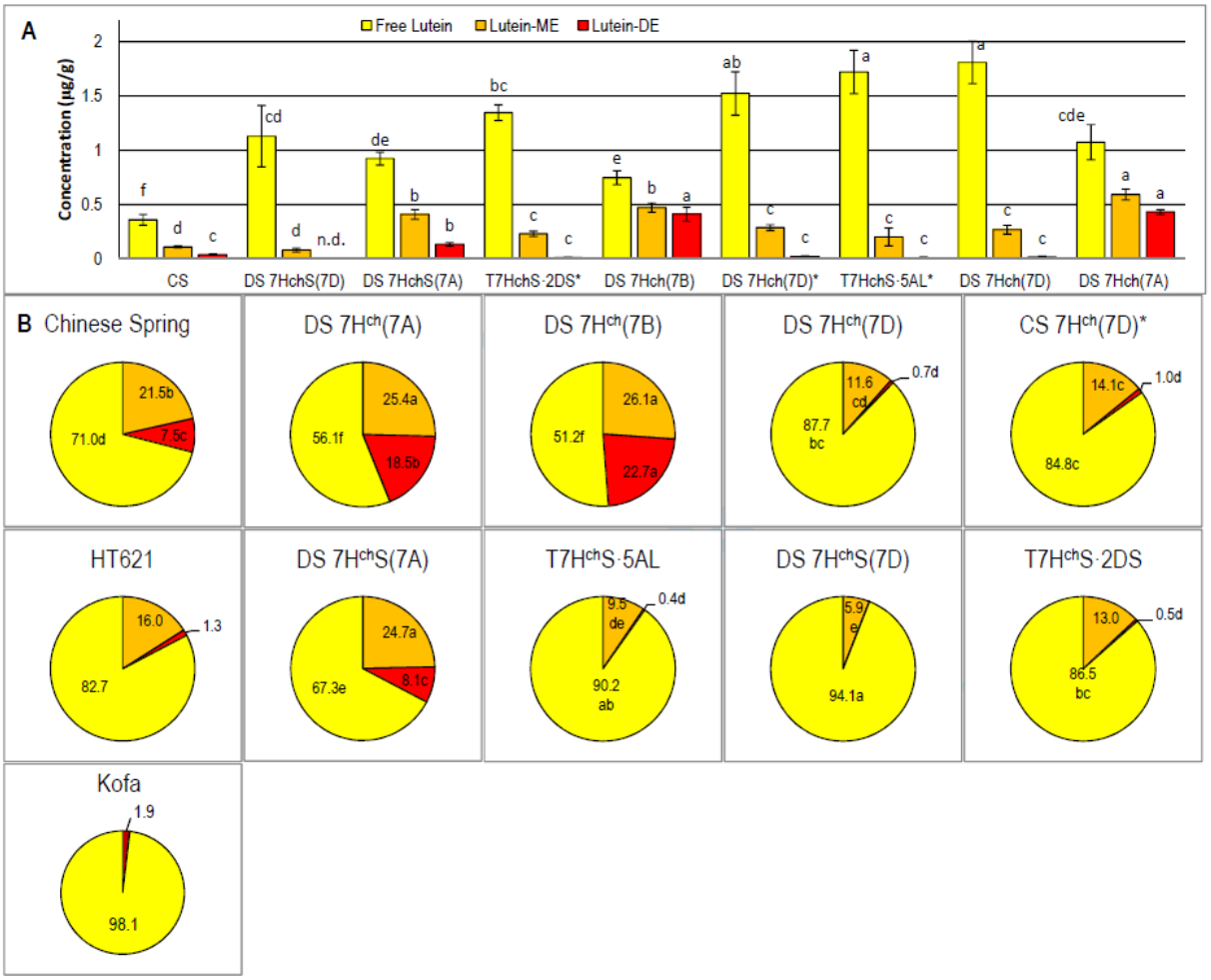

Fig 4. Lutein monoesters (lutein-ME) profile in the genetic stocks with improved carotenoid content. A. Contents of lutein monolinoleate and lutein monopalmitate. B. Relative contribution of each monoester (%) to the total content of lutein-ME. Tritordeum 'HT621' is shown only as reference since it was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

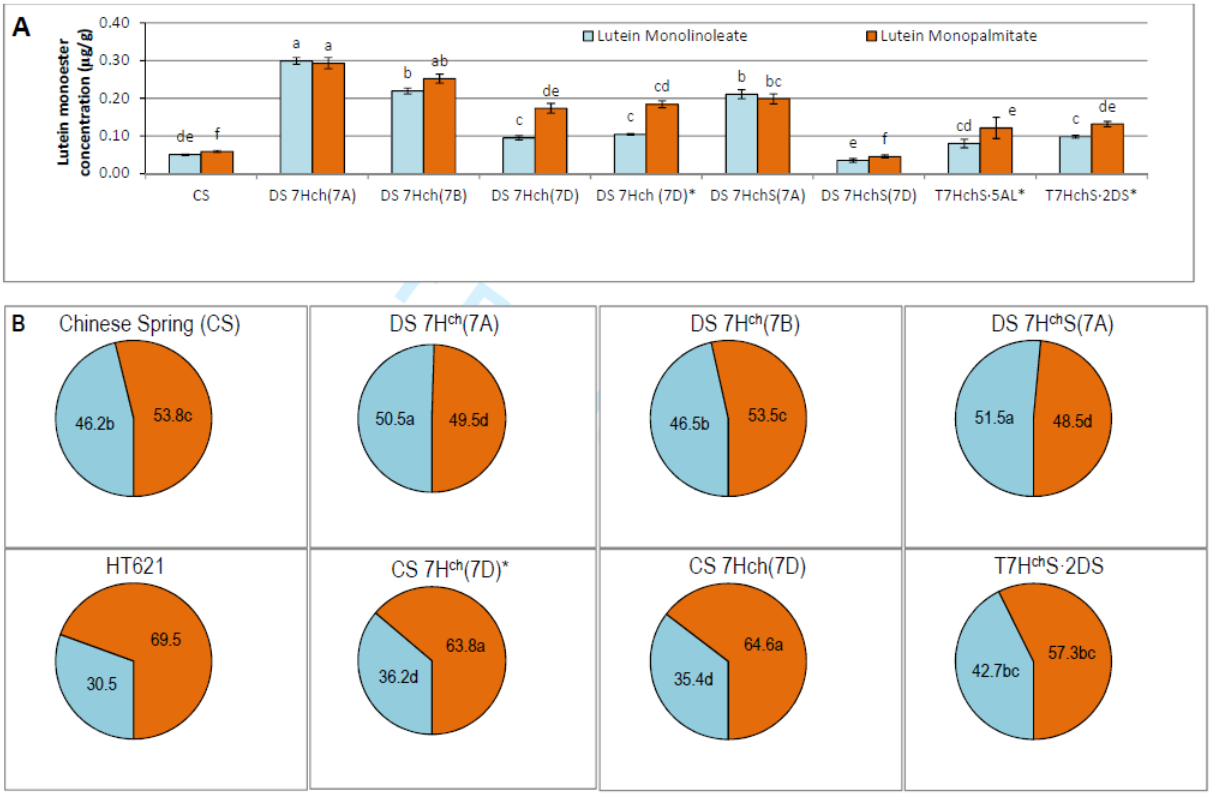

Fig 5. Lutein diester (lutein-DE) profile in the genetic stocks with improved carotenoid content relative to 'Chinese Spring'. A. Grain contents of lutein dilinoleate, lutein linoleate-palmitate and lutein dipalmitate. B. Relative contribution of each diester (%) to the total content of lutein-DE. Tritordeum '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.