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Lutein ester profile in wheat and tritordeum can be modulated by temperature:
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Running title: Temperature in grain filling alters carotenoids content and profile

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Abstract

The increase of lutein retention through the food chain is desirable for wheat breeding. Lutein esters are more stable than free lutein during post-harvest storage and two loci on chromosomes 7D and 7H^{ch} are important for esterification. We investigated the effect of temperature during grain filling on carotenoid accumulation and lutein ester profile including fatty acid selectivity (palmitic vs. linoleic) and regioselectivity (esterification at positions 3 vs. 3'). Three different temperature regimes were assayed (controlled, semi-controlled and non-controlled). Lutein esters were more stable than free carotenoids *in vivo* and the enzymes encoded by chromosomes 7H^{ch} and 7D are complementary. Indeed, they show differential preferences for the fatty acid (palmitic

and linoleic, respectively) and regioselectivity (3 and 3', respectively). Besides, *H. chilense* has additional genes for esterification. Finally, the increase of temperature favoured the accumulation of lutein esters with linoleic acid and the synthesis of regioisomers at position 3'.

Keywords:

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

1. Introduction

Carotenoid pigments, mainly lutein, are the main responsible for the yellow colour of wheat and related cereal seeds (reviewed by Rodríguez-Suárez, Giménez, & Atienza, 2010). Semolina production is associated with a bright yellow colour for pasta manufacturing which has promoted the enhancement of the lutein content in new durum wheat varieties (reviewed by Ficco, Mastrangelo, Trono, Borrelli, De Vita, Fares et al., 2014). Common wheat has been traditionally selected for white or pale creamy colour for bread although a stronger yellow colour may be beneficial for the production of yellow alkaline noodles (YAN) (Mares & Campbell, 2001). In fact, the contribution of lutein to yellowness of YAN has been proved to be much higher than other compounds such as apigenin di-C-glycoside (Wijaya, Ingram, Asenstorfer, & Mares, 2016). Moreover, new bread types with distinctive characteristics are being produced to satisfy consumer's demand. This has opened new market opportunities to species such as

tritordeum ($\times$ *Tritordeum* Aschers. et Graeb.). Hexaploid tritordeum is the amphiploid derived from the cross between a wild barley (*Hordeum chilense* Roem. et Schultz.) and durum wheat (*Triticum turgidum* spp. *durum*). At the moment tritordeum is being successfully commercialized (Vivagram®, Agrasys S.L.) due to its functional properties and golden colour among other characteristics (<http://tritordeum.com>). Similarly, bread derived from einkorn (*Triticum monococcum* L.) or khorasan wheat would also show a yellowish colour due to its high lutein content (Shewry & Hey, 2015).

Lutein is the main carotenoid present in wheat and tritordeum (reviewed by Ficco et al., 2014; Rodríguez-Suárez et al., 2010). The identification and mapping of carotenogenic genes in *H. chilense* (Rodríguez-Suárez & Atienza, 2012) has allowed the determination of the genetic basis of carotenoid content in tritordeum (Atienza, Avila, & Martin, 2007a; Rodríguez-Suárez, Mellado-Ortega, Hornero-Méndez, & Atienza, 2014). As observed in wheat, *Phytoene synthase 1* enzymes are the main responsible of the high lutein content in tritordeum and durum wheat grains (Ficco et al., 2014; Rodríguez-Suárez et al., 2010). Besides, a high proportion of lutein is esterified with fatty acids in tritordeum, including both lutein monoesters and diesters, whereas in durum wheat very low amounts of lutein monoesters are detected (Atienza, Ballesteros, Martin, & Hornero-Mendez, 2007b; Rodríguez-Suárez et al., 2014).

Lutein esters are more stable than free lutein (Subagio, Wakaki, & Morita, 1999). Similar findings have been found during post-harvest storage of cereal grains (Ahmad, Asenstorfer, Soriano, & Mares, 2013; Mellado-Ortega, Atienza, & Hornero-Méndez, 2015; Mellado-Ortega & Hornero-Méndez, 2016b). This is important since the losses of lutein can be significant during the storage of flour especially at high temperatures (Hidalgo & Brandolini, 2008) and during pasta and bread preparation (Hidalgo, Brandolini, & Pompei, 2010). High lipoxygenase (LOX) activity increases carotenoid

losses (Hidalgo & Brandolini, 2012; Leenhardt, Lyan, Rock, Boussard, Potus, Chanliaud et al., 2006) with an optimum temperature for the wheat LOX of 35°C (Sun, Du, Jin, Liu, Kong, 2012). Esterification is an interesting characteristic to improve the retention of lutein through the food chain since it increases lutein stability at high temperatures. Thus, a better understanding of lutein ester formation is important in order to improve lutein retention in cereal-derived products.

H. chilense is responsible for the high carotenoid content of tritordeum and for its carotenoid esterification pattern (Mellado-Ortega & Hornero-Méndez, 2015). Previous studies have shown a lack of correlation between the fatty acid profile of the total lipid fraction and the fatty acid involved in the esterification of xanthophylls, suggesting that the enzymes responsible for the formation of xanthophyll esters (xanthophyll acyl transferases, XAT) are highly selective regarding the fatty acid (Breithaupt & Schwack, 2000; Delgado-Pelayo, Gallardo-Guerrero, & Hornero-Mendez, 2016; Delgado-Pelayo & Hornero-Mendez, 2012; Fernandez-Orozco, Gallardo-Guerrero, & Hornero-Mendez, 2013; Mellado-Ortega & Hornero-Mendez, 2012). Recent findings in our labs have demonstrated the existence of at least two genes for lutein esterification in chromosomes 7D and 7H^{ch} (Mattera, Cabrera, Hornero-Méndez, & Atienza, 2015b). The enzyme from 7H^{ch} shows a preferential esterification of lutein with palmitic acid (Mattera et al., 2015b; Mellado-Ortega & Hornero-Mendez, 2012), while the enzyme from 7D does not show a clear difference for the preferential use of palmitic and linoleic acids (Mattera et al., 2015b). The existence of a gene for lutein esterification in chromosome 7D has been independently proved in common wheat (Ahmad, Mather, Law, Li, Yousif, Chalmers et al., 2015) after 60 days of storage at 37°C. Besides, an important positive effect of temperature on the esterification of lutein during post-

harvest storage of grains and flours has been proven (Ahmad et al., 2013; Mellado-Ortega et al., 2015; Mellado-Ortega & Hornero-Méndez, 2016a).

Based on the important role of temperature on lutein esterification during post-harvest storage, it is worth investigating its effect during grain filling in carotenoid accumulation and esterification. In particular we aimed to investigate the effect of temperature during grain filling on carotenoid content and lutein ester profile, including the effect on the selectivity for the use of palmitic and linoleic acids along with regioselectivity (esterification at positions 3 and 3').

2. Materials and Methods

2.1. Plant materials

The plant materials used in this work included the following lines: Two hexaploid tritordeum lines, HT609 and HT621, selected at Institute for Sustainable Agriculture – CSIC (Córdoba, Spain). HT621 was registered as high YPC line (Ballesteros, Ramirez, Martinez, Atienza, & Martin, 2005). A high lutein common wheat line, DM5685*B12, developed at the University of Adelaide (Australia), (seeds were kindly supplied by Prof. D. Mares, University of Adelaide). A durum wheat variety, 'Kofa' (PI584336) developed in Arizona, US. The common wheat genotype 'Chinese Spring' (CS). Three wheat-*H. chilense* chromosome substitution lines [DS 7H^{ch}(7A), DS 7H^{ch}(7B), DS 7H^{ch}(7D), where DS means disomic substitution] developed at John Innes Center, Norwich, UK (T.E. Miller and S.M. Reader, unpubl.results;

www.jic.ac.uk/germplasm/Wheat-Precise-Genetic_stocks-Aliens.pdf), and an additional line [DS 7H^{ch}(7D)] developed at University of Córdoba, Spain (Mattera, Ávila, Atienza, & Cabrera, 2015a).

2.2. Experimental conditions

All genotypes were grown in 1L pots with a mixture 1:4.5 (v:v) (sand:premium pflanzerde substrate). Osmocote Exact Mini (1.2 g/L) (Everris International B.V.) was added. Seeds were germinated in the greenhouse and then transferred outdoors. Pods were grown on anti-weeds nets under an anti-trip net structure but without further sheltering against meteorological conditions. At heading, plants of each accession were randomly selected to develop three experimental trials with different control of temperature: growing chamber, greenhouse and outdoor conditions. Each trial was established using a completely randomized block design with three replications. Temperature was controlled in growth chamber [light period: 24°C during 11 hours + 19°C during 1 hour; dark period: 14°C during 11 hours + 19°C during 1 hour]. A cooling system was used within the greenhouse and setup to be activated at 27°C to avoid reaching very high temperatures during the daylight. Finally, no temperature control was conducted in the experiment performed outdoors. Temperature was monitored each 20 minutes using dataloggers (EBI 20-TH1, Ebro, Germany). At maturity, seeds were harvested and maintained at 4°C during four months until further processing.

2.3. Extraction of carotenoids

Carotenoid pigments were extracted from grains using the method described by Mellado-Ortega & Hornero-Méndez (2016a), with slight modifications. One gram of

grain sample with 6 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 balls (10 mm Ø) at 25 Hz for 1 min. All samples were milled in duplicate and a known amount of internal standard (canthaxanthin, 1.5 µg) 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 residue containing the pigments was dissolved in 0.5 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.

2.4. HPLC analyses of carotenoids

The procedures for the identification of carotenoid pigments and their esters (including regioisomers) in cereal grains have already been described in previous works (Atienza et al., 2007b; Mellado-Ortega & Hornero-Mendez, 2012).

Quantitative HPLC analysis of carotenoids was carried out according to the method of Mínguez-Mosquera & 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 µm, 20×0.46 cm; Teknokroma, Barcelona, Spain) was used. Pigment 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 & 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. Therefore the concentration of lutein esters was expressed as free lutein equivalents. 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.

2.5. Statistical analyses

Analyses of variance for total carotenoid content and carotenoid profile were performed using Statistix version 9.0 (Analytical Software, Tallahassee, FL, USA). Differences among genotypes or growing conditions were established using Tukey's honest significance (HSD) test at $P < 0.05$.

3. Results

A detailed analysis of the carotenoid composition including carotenoid esters was carried out by HPLC (Table 1). Lutein (mainly the all-*trans* isomer, with small amounts of 13-*cis* and 9-*cis* isomers) was the major carotenoid pigment detected in all samples in agreement with previous findings (Mattera et al., 2015b; Mellado-Ortega et al., 2015; Mellado-Ortega & Hornero-Mendez, 2012). Lutein accounted for 84-96% of total carotenoid content together with minor amounts of β -carotene and all-*trans*-zeaxanthin

(Table 1). Lutein monesters and lutein diesters were also found and quantified in most samples. The unique exception was 'Kofa', where traces of lutein monoesters and no lutein diesters were detected in agreement with previous results in durum wheat (Atienza et al., 2007b; Mellado-Ortega et al., 2015).

All the genotypes had a higher total carotenoid content in grain than the common wheat variety 'Chinese Spring' (CS) (Fig. 1A). Tritordeum lines HT609 and HT621 contained the highest amounts of lutein (sum of free and esterified forms) and total carotenoids. The high-lutein wheat DM5685*B12, kindly provided by Prof. Daryl Mares (University of Adelaide, Australia), reached similar carotenoid content than durum wheat 'Kofa'. The four disomic substitution lines developed in CS background [DS 7H^{ch}(7A), DS 7H^{ch}(7B), and two DS 7H^{ch}(7D) lines from different origins] also showed similar carotenoid content to Kofa, due to the presence of *Psy1* from *H. chilense* (Fig. 1A). Besides, HT621, HT609, DM5685*B12, DS 7H^{ch}(7A) and DS 7H^{ch}(7B) showed the highest amounts of lutein esters (Fig. 1A).

Growing conditions affected total carotenoid content and profile. The lowest amounts of total carotenoid, total lutein and free lutein were obtained at outdoor conditions (Fig. 1B), where no control of temperature was established. Fig. 1B shows a clear decreasing tendency for free lutein with growing chamber > greenhouse > outdoor conditions. As planned, the experimental conditions resulted in differences in average daily temperatures among the different trials. In growth chamber, a steady average temperature around 16 °C was observed during the complete growing cycle (Supplemental Fig. 1). On the contrary, average daily temperature increased along the grain filling period in both greenhouse (from 17 to 23°C) and outdoors experiments (from 17 to 26.6°C) from the beginning of the experiment to the day before harvest (Supplemental Fig. 1). Besides, maximum temperatures reached outdoors were

significantly higher than in the glasshouse (Supplemental Fig. 2). Plants grown outdoors matured three days earlier than those maintained at greenhouse while plants kept in the growth chamber required almost three additional weeks.

Total carotenoids and lutein contents were reduced with the increase of temperature (Fig. 1B) but lutein esterification did not show a clear tendency. Indeed, the highest contents of lutein esters were obtained in greenhouse, the lowest in growth chamber and intermediate levels at outdoor conditions (Fig. 1B).

In agreement with previous works (Mellado-Ortega et al., 2015; Mellado-Ortega & Hornero-Mendez, 2012), lutein monoester fraction was composed of lutein linoleate and lutein palmitate, while lutein diesters comprised lutein dilinoleate, lutein linoleate-palmitate and lutein dipalmitate. Furthermore, we identified the two possible regioisomeric forms for each of the lutein monoesters (Fig. 2). Both tritordeum lines along with both chromosome substitution lines (7D) 7H^{ch} preferentially formed the monoester regioisomer at the hydroxyl group at position 3 of the lutein molecule [lutein 3-*O*-linoleate (Fig. 3A) and lutein 3-*O*-palmitate (Fig. 3B)]. The ratio between regioisomers of lutein monoesters at position 3 and 3' was about 3.5:1 for lutein monolinoleate (Fig. 3A) and about 2:1 for lutein monopalmitate (Fig. 3B).

The remaining genotypes (CS, CS (7A)/7H^{ch}, CS (7B)/7H^{ch} and DM5685*B12), all of them carrying chromosome 7D, were significantly different from the previous ones for the 3/3' regioisomer ratio. Indeed, all of them showed a ratio below 1 for lutein monopalmitate (Fig. 3B) which indicates that esterification takes place preferentially at the hydroxyl group at 3' position. Differences were also evident for lutein monolinoleate regioisomers (Fig. 3A). Growing conditions also affected the balance between regioisomers (Fig. 3C and 3D). The 3/3' ratio decreased with temperature

(growing chamber > greenhouse > outdoors) which seems to indicate that esterification at position 3' is enhanced with temperature.

Five lines showed similar levels of lutein esters (HT609, HT621, DM5685*B12, DS (7A)/7H^{ch} and DS (7B)/7H^{ch}). Thus, we examined the lutein ester profile of these lines to clarify the effect of temperature on lutein esterification during grain filling.

Tritordeum lines synthesized higher amounts of lutein monopalmitate while both monoesters yielded similar quantities in DM5685*B12, DS (7A)/7H^{ch} and DS (7B)/7H^{ch} (Fig. 4A). The comparison of the different growing conditions did not show a clear tendency. HT609 and DS (7B)/7H^{ch} did not show any significant variation for lutein monopalmitate or lutein monolinoleate among the growing conditions tested (Fig. 4A). Significant differences for lutein monolinoleate were observed in both HT621 and DS (7A)/7H^{ch} but it was not possible to establish a clear tendency. Finally, the high lutein wheat DM5685*B12 increased the amounts of both lutein monopalmitate and lutein monolinoleate, although differences for lutein monopalmitate were not significant at $P < 0.05$.

Tritordeum lines preferentially esterified lutein with palmitic acid for the synthesis of lutein diesters (Fig. 4B). In contrast DM5685*B12, DS (7A)/7H^{ch} and DS (7B)/7H^{ch} did not show evidences of preferential esterification towards any fatty acid as suggested by the similar levels of lutein dilinoleate and lutein dipalmitate for each genotype in each growing condition (Fig. 4B).

HT609, HT621 and DM5685*B12 did not show significant differences related to growing conditions for any of these esters (Fig. 4B). However, both DS(7A)/7H^{ch} and DS(7B)/7H^{ch} increased the amounts of individual lutein diesters with the increase of temperature (Fig. 4B).

In order to investigate whether temperature favours or modulate the esterification with linoleic acid or with palmitic acid, the following ratio was calculated $[(\text{lutein monolinoleate} + \text{lutein dilinoleate}) / (\text{lutein monopalmitate} + \text{lutein dipalmitate})]$. A higher ratio was obtained in both greenhouse and outdoor conditions compared to growth chamber (Fig. 5) which indicates that the use of linoleic acid as substrate for lutein esterification was enhanced when daily temperature was higher.

4. Discussion

Plants grown at outdoor conditions showed the lowest contents for total carotenoid, total lutein and free lutein. Temperature regime was the main difference among the three growing conditions. Indeed, plants grown in the greenhouse and outdoors received the same light conditions. However, the cooling system prevented the occurrence of high temperatures in the greenhouse in contrast with the outdoors experiment where no mitigating measures were established. Thus, the lower carotenoid content can be associated with the higher temperatures, which also induced a faster rate of grain development in agreement with previous findings (reviewed by Maestri, Klueva, Perrotta, Gulli, Nguyen, & Marmiroli, 2002). On the other hand, the higher carotenoid content of plants in the growth chamber might be related to the longer grain filling period, although it did not allow for higher amounts of lutein esters.

The reduction of the carotenoid content due to high temperature could be explained by a higher metabolism rate, a lower carotenoid synthesis or both. Total carotenoids content in common wheat flag leaves increases due to heat stress (Dias, Semedo, Ramalho, & Lidon, 2011) which indicates the carotenoid synthesis is enhanced with temperature.

Besides carotenoid metabolism is induced in rice bran during postharvest yellowing due

to high temperature stress (Belefant-Miller & Grunden, 2014). Thus, it is plausible that lutein is being metabolized as a consequence of high temperatures in wheat and tritordeum during grain filling.

The incidence of heat stress during photosynthesis increases the rate of production of reactive oxygen species (ROS) (reviewed by Suzuki & Mittler, 2006). The very high maximum temperatures reached outdoors will promote the formation of ROS which would reduce the carotenoid content since carotenoids are used to detoxify ROS (Howitt & Pogson, 2006). Thus, it is likely that the lower carotenoid content of plants grown at outdoors compared to greenhouse is due to a higher metabolism rate.

All the chromosome substitution lines used in this work have been developed in CS background and they showed the highest differences in total carotenoid content between outdoor and growth chamber conditions. Chinese Spring is sensitive to heat stress (Qin, Wu, Peng, Yao, Ni, Li et al., 2008) which implies that a higher quantity of ROS is produced under heat conditions (Suzuki & Mittler, 2006). Thus, this would explain the reduction in carotenoid content observed in CS and the chromosome substitution lines in greenhouse and outdoors experiments. On the contrary, tritordeum genotypes, Australian line DM5685*B12, and US durum wheat 'Kofa' showed lower differences between growth chamber and outdoor conditions since they are better adapted to our climate. Indeed, HT609 and HT621 have been locally selected for agronomic performance, and thus, they are well adapted to high temperatures during grain filling. Similarly, DM5685*B12 was developed at Adelaide (South Australia) and it was selected for lutein and other quality and agronomic traits (Ahmad et al., 2013). Since Adelaide has also a Mediterranean climate, this line was not expected to be heat sensitive either. Finally, 'Kofa' is a Desert Durum® variety developed by Western Plant Breeders at Arizona (US) and it has been used as parental line for the development

of the wheat CAP population Kofa/UC1113 (Zhang, Chao, Manthey, Chicaiza, Brevis, Echenique et al., 2008) due to excellent pasta quality and with optimal semolina and pasta colour. The higher heat tolerance of these lines is consistent with a lower production of ROS or with a higher amount of antioxidant compounds and thus, with a lower decay of carotenoid content in grain.

Lutein esters were only produced using linoleic and palmitic acid as substrates in agreement with previous results (Ahmad et al., 2013; Mattera et al., 2015b; Mellado-Ortega et al., 2015; Mellado-Ortega & Hornero-Mendez, 2012). Recent findings have also reported lutein esterification with oleic acid in bread wheat, spelt (*T. spelta* L.) and einkorn (*T. monococcum* L.) (Ziegler, Wahl, Würschum, Longin, Carle, & Schweiggert, 2015). So far, we have detected only traces of lutein esters with oleic acid in tritordeum or *H. chilense* (unpublished results) but genetic diversity for these compounds may be important in other species.

Previous findings suggested that the xanthophyll acyltransferase enzyme involved in the esterification of carotenoids has a preferential acylating action over the β -end ring of lutein compared to the ϵ -end ring in tritordeum (Mellado-Ortega & Hornero-Mendez, 2012). *H. chilense* is responsible for the esterification profile of tritordeums (Mellado-Ortega & Hornero-Méndez, 2015) with gene(s) located on chromosome 7H^{ch} playing a significant role (Mattera et al., 2015b). Our data confirm that the enzyme derived from *H. chilense* genome has preference over the β -ring. On the contrary, genotypes carrying chromosome 7D seem to favour acylation on ϵ -ring. Thus, the enzymes derived from chromosomes 7D and 7H^{ch} could be considered as complementary. These enzymes may be variants of the same enzyme (known as allozymes) or they could be totally different enzymes. In any case, they differ for their optimum reaction conditions. Indeed, the ratio between regioisomers of lutein monoesters at position 3 and 3' was above 1 in

most growing conditions (Fig. 3C, 3D) which indicates that the β -ring is preferentially used for lutein esterification. However, this ratio decreased with the increase in temperature which indicates that acylation at position 3' becomes more efficient.

Lutein esterification is enhanced during post-harvest storage with increasing temperature (Ahmad et al., 2013; Mellado-Ortega et al., 2015). Besides, esterification proceeds faster when the relative humidity is lower (Kaneko & Oyanagi, 1995). Collectively, our data do not show a clear trend of lutein ester accumulation with increasing temperature. Remarkably, the amounts of lutein esters were not significantly reduced in outdoor conditions (Fig. 1B). This supports that lutein esters are more stable than free lutein which showed a clear decreasing pattern (Fig. 1B) as shown in previous works. Thus, esterification is an interesting trait for the improvement of the retention of lutein in wheat derived products (Ahmad et al., 2015; Mellado-Ortega & Hornero Méndez, 2016b).

The individual analysis of genotypes with significant amounts of lutein esters (Fig. 4) shows a clearer picture. Lutein diesters were enhanced with increasing temperature in both DS 7H^{ch} (7A) and DS 7H^{ch} (7B). Both genotypes carry chromosome 7D as happens with DM5685*B12 which showed higher lutein monoesters content in greenhouse and outdoor conditions compared to growth chamber (Fig. 4A). The same trend was appreciated for lutein diesters in DM5685*B12, but in this case, the observed differences were not significant after ANOVA (Fig. 4B).

Interestingly, both tritordeums lines did not show significant differences for any lutein diester (Fig. 4A). It is important to note that hexaploid tritordeums carry the genomes A, B and H^{ch}. Thus, they lack genome 7D. Our previous studies have demonstrated that lutein is preferentially esterified with palmitic acid in tritordeum (Mattera et al., 2015b;

Mellado-Ortega & Hornero-Mendez, 2012), despite linoleic acid is more abundant in the lipid pool containing the acyl-donors. This implies the existence of substrate specificity. Recent findings have demonstrated the existence of loci for lutein esterification in chromosomes 7D (Ahmad et al., 2015) and 7D and 7H^{ch} (Mattera et al., 2015b), although the existence of other loci influencing this trait cannot be discarded. For instance, the first QTL for lutein esterification was located in chromosome 2B (Howitt, Cavanagh, Bowerman, Cazzonelli, Rampling, Mimica et al., 2009). We have proven that the enzyme(s) encoded by locus (or loci) in chromosome 7H^{ch} shows specificity towards palmitic acid for lutein esterification while chromosome 7D is either indifferent or it prefers linoleic acid (Mattera et al., 2015b). Our current results corroborate these findings.

Genotypes carrying chromosome 7D showed an enhanced lutein ester content compared to tritordeum lines with increasing temperature. Thus, we investigated whether lutein was equally esterified with both palmitic and linoleic acids in the three experimental conditions. Interestingly, the ratio $\sum \text{linoleic esters} / \sum \text{palmitic esters}$ increased with temperature (Fig. 5), which indicates that esterification with linoleic acid was benefited by the increase of temperature (Fig. 5). Recent findings have suggested that the enzyme responsible for lutein esterification in DM5685*B12 is very thermostable with an optimum temperature at 80°C (Ahmad et al., 2013). The preferential esterification with linoleic acid suggests an improvement of the conditions for the esterification reaction. Whether it is due to a better performance of the enzyme for lutein esterification or a higher availability of linoleic acid (or the corresponding acyl-donor) at the site of the esterification reaction cannot be discerned at present.

5. Conclusions

A high temperature regime during grain filling results in lower total carotenoid and free lutein contents. However, lutein esters did not show significant variations which prove that they are more stable than free carotenoids *in vivo*. Thus, a higher esterification capacity would be desirable to increase lutein retention for the production of wheat-derived products such as pasta, yellow-alkaline noodles or high-lutein bread. The enzymes encoded by chromosomes 7H^{ch} and 7D are complementary since they show differential preferences for the fatty acid (palmitic and linoleic acid respectively) and the position (3 and 3' respectively, regioselectivity). This may explain the higher levels of lutein diesters when both 7D and 7H^{ch} chromosomes are simultaneously present. In any case, tritordeum lines synthesize higher amounts of lutein esters which indicates that *H. chilense* genome possess other genes important for lutein esterification. Finally, the occurrence of higher temperatures during grain filling favoured the accumulation of lutein esters with linoleic acid and the synthesis of regioisomers at position 3'.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Caption figures

Fig. 1. Carotenoid content and profile ($\mu\text{g/g}$ dry weight) in grain. (A) Effect of genotype. Each bar represents the average among the three growing conditions (trials). For each compound, bars with the same letter are not significantly different at $P < 0.05$, determined by Tukey's HSD test. DS 7H^{ch}(7D)* was described by (Mattera et al., 2015a). (B) Effect of growing conditions. Each bar represents the average considering all the genotypes. For each compound, bars with the same letter are not significantly different at $P < 0.05$, determined by Tukey's HSD test. TotCar = Total carotenoids; TotLut = Total lutein; Free Lut = Free lutein; TotLutEs = Total esterified lutein.

Fig. 2. Chemical structures of the lutein monoesters, including regioisomers, identified in this work.

Fig. 3. Effect of genotype and growing conditions on lutein ester regioisomers profile. (A) Relative contributions of lutein monolinoleate regioisomers; (B) Relative contribution of lutein monopalmitate regioisomers; (C) Differences in lutein monolinoleate profile due to growing conditions; (D) Differences in lutein monopalmitate profile due to growing conditions. DS 7H^{ch}(7D)* was described by Mattera et al., (2015a). Numbers above each bar represent the ratio between regioisomers of lutein monoesters at position 3 and 3'. For each case, ratios with the same letter are not significantly different at $P < 0.05$, determined by Tukey's HSD test.

Fig. 4. Lutein ester profile in selected genotypes with the higher amounts of lutein diester. A. Contents of lutein monoesters. B. Contents of lutein diesters. For each compound and genotype, differences among growing conditions (GC = growing chamber; GH = greenhouse; OD = Outdoors) were determined using Tukey's HSD test. Bars with the same letter are not significantly different at $P < 0.05$.

Fig. 5. Effect of temperature on the balance of lutein esters. Evolution of the ratio $\Sigma(\text{lutein monolinoleate} + \text{lutein dilinoleate}) / \Sigma(\text{lutein monopalmitate} + \text{lutein dipalmitate})$. Bars with the same letter are not significantly different at $P < 0.05$.

Supplemental Fig. 1. Average daily temperature from heading to maturity of grains under the three growing conditions.

Supplemental Fig. 2. Maximum daily temperature from heading to maturity of grains.

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A

B

R1		R2
(β-end ring)	Monoester	(ε-end ring)
Palmitoyl	Lutein-3- <i>O</i> -palmitate	H
H	Lutein-3'- <i>O</i> -palmitate	Palmitoyl
Linoleoyl	Lutein-3- <i>O</i> -linoleate	H
H	Lutein-3'- <i>O</i> -linoleate	Linoleoyl

Table 1. Carotenoid composition in mature grains grown at different environmental conditions expressed in $\mu\text{g/g}$ fresh weight

	Genotype ¹	all- <i>trans</i> -zeaxanthin	all <i>trans</i> - β -carotene	Free lutein ²	Lutein monoester ³	Lutein diester ⁴	Total lutein ⁵	Total carotenoids ⁶
Growth chamber	DS 7H ^{ch} (7D)*	0.16 $\pm$ 0.02	0.10 $\pm$ 0.00	3.00 $\pm$ 0.08	0.24 $\pm$ 0.02	0.01 $\pm$ 0.00	3.25 $\pm$ 0.11	3.51 $\pm$ 0.12
	HT609	0.16 $\pm$ 0.01	0.08 $\pm$ 0.00	5.20 $\pm$ 0.22	0.70 $\pm$ 0.09	0.09 $\pm$ 0.02	6.00 $\pm$ 0.31	6.24 $\pm$ 0.32
	HT621	0.19 $\pm$ 0.01	0.05 $\pm$ 0.02	5.07 $\pm$ 0.68	0.58 $\pm$ 0.00	0.06 $\pm$ 0.01	5.71 $\pm$ 0.79	5.95 $\pm$ 0.81
	Kofa	0.16 $\pm$ 0.00	0.05 $\pm$ 0.00	3.15 $\pm$ 0.02	0.02 $\pm$ 0.00	ND ⁷	3.18 $\pm$ 0.03	3.38 $\pm$ 0.03
	DM56							
	85*B1			2.61 $\pm$ 0.11	0.37 $\pm$ 0.05	0.06 $\pm$ 0.02	3.05 $\pm$ 0.15	3.29 $\pm$ 0.14
	2	0.17 $\pm$ 0.02	0.07 $\pm$ 0.01	0.75 $\pm$ 0.06	0.10 $\pm$ 0.01	0.03 $\pm$ 0.00	0.88 $\pm$ 0.07	1.00 $\pm$ 0.07
	CS	0.10 $\pm$ 0.01	0.01 $\pm$ 0.00	0.06	1	00	0.07	7
	DS 7H ^{ch} (7A)	0.15 $\pm$ 0.00	0.14 $\pm$ 0.02	2.44 $\pm$ 0.16	0.51 $\pm$ 0.02	0.18 $\pm$ 0.01	3.13 $\pm$ 0.18	3.42 $\pm$ 0.20
	DS 7H ^{ch} (7B)	0.13 $\pm$ 0.00	0.05 $\pm$ 0.00	1.75 $\pm$ 0.05	0.51 $\pm$ 0.03	0.29 $\pm$ 0.01	2.55 $\pm$ 0.08	2.74 $\pm$ 0.08
	DS 7H ^{ch} (7D)	0.15 $\pm$ 0.01	0.09 $\pm$ 0.00	2.83 $\pm$ 0.12	0.38 $\pm$ 0.03	0.04 $\pm$ 0.00	3.26 $\pm$ 0.16	3.50 $\pm$ 0.16
Greenhouse	DS 7H ^{ch} (7D)*	0.22 $\pm$ 0.02	0.09 $\pm$ 0.02	2.47 $\pm$ 0.37	0.30 $\pm$ 0.05	0.02 $\pm$ 0.00	2.79 $\pm$ 0.42	3.10 $\pm$ 0.45
	HT609	0.16 $\pm$ 0.01	0.07 $\pm$ 0.01	4.44 $\pm$ 0.64	0.71 $\pm$ 0.07	0.10 $\pm$ 0.01	5.26 $\pm$ 0.72	5.49 $\pm$ 0.74
	HT621	0.19 $\pm$ 0.01	0.07 $\pm$ 0.01	5.01 $\pm$ 0.15	0.73 $\pm$ 0.05	0.09 $\pm$ 0.01	5.83 $\pm$ 0.20	6.09 $\pm$ 0.20
	Kofa	0.14 $\pm$ 0.00	0.03 $\pm$ 0.00	2.36 $\pm$ 0.17	0.03 $\pm$ 0.00	ND	2.39 $\pm$ 0.18	2.56 $\pm$ 0.18
	DM56							
	85*B1			3.26 $\pm$ 0.36	0.57 $\pm$ 0.03	0.09 $\pm$ 0.01	3.92 $\pm$ 0.38	4.16 $\pm$ 0.40
	2	0.15 $\pm$ 0.01	0.09 $\pm$ 0.02	0.53 $\pm$ 0.06	0.11 $\pm$ 0.01	0.05 $\pm$ 0.00	0.69 $\pm$ 0.07	0.80 $\pm$ 0.08
	CS	0.09 $\pm$ 0.01	0.01 $\pm$ 0.00	0.06	1	00	0.07	8
	DS 7H ^{ch} (7A)	0.17 $\pm$ 0.01	0.11 $\pm$ 0.02	1.88 $\pm$ 0.30	0.62 $\pm$ 0.00	0.33 $\pm$ 0.05	2.83 $\pm$ 0.45	3.11 $\pm$ 0.47
	DS 7H ^{ch} (7B)	0.14 $\pm$ 0.01	0.05 $\pm$ 0.02	1.07 $\pm$ 0.33	0.45 $\pm$ 0.09	0.37 $\pm$ 0.04	1.90 $\pm$ 0.45	2.08 $\pm$ 0.48
	DS 7H ^{ch} (7D)	0.17 $\pm$ 0.01	0.07 $\pm$ 0.01	2.37 $\pm$ 0.14	0.31 $\pm$ 0.02	0.03 $\pm$ 0.00	2.71 $\pm$ 0.16	2.95 $\pm$ 0.17

D)								
Outdoor s	DS							
	7H ^{ch} (7			1.93±	0.21±0.0	0.02±0.	2.14±	2.36±0.3
	D)*	0.18±0.01	0.03±0.02	0.26	3	00	0.30	2
				4.37±	0.63±0.0	0.09±0.	5.08±	5.33±0.3
	HT609	0.19±0.01	0.06±0.01	0.34	3	01	0.37	8
				4.47±	0.63±0.0	0.09±0.	5.18±	5.44±0.6
	HT621	0.20±0.01	0.06±0.01	0.53	5	00	0.58	1
				2.29±	0.03±0.0		2.31±	2.51±0.0
	Kofa	0.17±0.00	0.02±0.00	0.10	1	ND	0.09	9
	DM56							
	85*B1			2.04±	0.54±0.0	0.13±0.	2.72±	2.93±0.1
	2	0.16±0.01	0.05±0.00	0.17	4	02	0.15	6
				0.36±	0.11±0.0	0.06±0.	0.53±	0.63±0.0
	CS	0.09±0.00	0.01±0.00	0.01	1	00	0.02	3
	DS							
	7H ^{ch} (7			0.79±	0.43±0.0	0.35±0.	1.57±	1.80±0.1
	A)	0.14±0.01	0.09±0.03	0.15	6	07	0.14	0
	DS							
	7H ^{ch} (7			0.78±	0.42±0.0	0.46±0.	1.67±	1.82±0.0
	B)	0.12±0.01	0.03±0.00	0.10	2	05	0.07	9
	DS							
	7H ^{ch} (7			1.92±	0.20±0.0	0.01±0.	2.13±	2.34±0.1
	D)	0.17±0.01	0.04±0.01	0.11	2	01	0.13	4

Data are mean ± SE. ²Free Lutein = all-*trans*-Lutein + 9-*cis*-Lutein + 13-*cis*-Lutein.

³Lutein monoester = Lutein monolinoleate + Lutein monopalmitate. ⁴Lutein diester = Lutein dilinoleate + lutein linoleate-palmitate + Lutein dipalmitate. ⁵Total lutein includes free lutein, lutein monoester and lutein diester. ⁶Total Carotenoids = all-*trans*-Zeaxanthin + all-*trans*-β-Carotene + total lutein. ⁷ND: not detected

Highlight

Lutein esters are more stable than free lutein *in vivo*.

Acetyltransferases encoded by genes in chromosomes 7D and 7H^{ch} are complementary.

XAT enzymes show marked regioselectivity for lutein esterification.

Higher temperature during grain filling modifies the lutein esterification profile.

H. chilense genome seems to carry additional genes for lutein esterification.

ACCEPTED MANUSCRIPT