Title: Carotenoid content in tritordeum is not primarily associated with esterification during grain development

Running title: Lutein esters and carotenoid accumulation in tritordeum

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Abstract

Tritordeums show a significant proportion of lutein esters which increases carotenoid stability and retention throughout the food chain. Esterification is a common means of carotenoid sequestration. A putative association between lutein esters formation acting as a metabolic sink during early stages of grain development and the high carotenoid content of tritordeums is analyzed in this work.

Compared to wheat, tritordeums accumulated significantly higher lutein contents from 20 days post anthesis (dpa) but lutein esters were not detected until 36 dpa. Thus esterification is not acting as a metabolic sink before 36dpa. The presence of lutein esters at late stages of grain development may have a complementary role in carotenoid accumulation by reducing and/or counteracting their catabolism. The differences for lutein esterification among tritordeums suggest the existence of diversity for xanthophyll acyl transferases that could be exploited to increase lutein retention in this cereal and through the food chain.

Keywords

Esterification; lutein esters; lutein; tritordeum; carotenoids;

1. Introduction

Carotenoids are isoprenoids produced by all photosynthetic organisms and some non-photosynthetic ones. They have essential functions in plants including photoprotection against photooxidative damage, play a role in the assembly of pigment-protein complexes and the electron flow cycle to chlorophyll in the reaction center of photosystem II, and they serve as precursors to hormones such as strigolactones and abscisic acid (ABA) (Rodríguez-Concepción, Avalos, Bonet, Boronat, Gomez-Gomez, Hornero-Mendez et al., 2018).

Carotenoids also play a significant role in human nutrition. Carotenoids with unsubstituted β -rings, such as β -carotene and β -cryptoxanthin, have provitamin A activity and they are considered essential nutrients since they cannot be synthesized *de novo* by humans (Fraser & Bramley, 2004). Other carotenoids such as zeaxanthin and lutein are also beneficial since their consumption may prevent age-related macular degradation (Carpentier, Knaus, & Suh, 2009). In addition, diets including carotenoid-rich foods have been associated with a lower risk of developing chronic diseases and certain types of cancer (Nishino, Murakoshi, Tokuda, & Satomi, 2009).

The importance of carotenoids at the nutritional level has promoted the development of biofortification strategies devised to increase the carotenoid content in staple crops such as maize by modifying the carotenoid pathway (Zhu, Naqvi, Breitenbach, Sandmann, Christou, & Capell, 2008) or using the natural variation in breeding programs of maize (Harjes, Rocheford, Bai, Brutnell, Kandianis, Sowinski et al., 2008). Besides carotenoids also play an important role in providing food color. In durum wheat, they provide the yellow pigment content (YPC) essential for pasta production (Ficco, Mastrangelo, Trono, Borrelli, De Vita, Fares et al., 2014) and thus carotenoid content, indirectly selected as YPC, has become an important selection criterion in durum wheat breeding programs. Moreover, in view of the increasing importance of health aspects in food quality, determining the carotenoid profile is considered useful for evaluating the

potential contribution to health of durum wheat end-products (Digesù, Platani, Cattivelli, Mangini, & Blanco, 2009).

The same happens with tritordeum (*×Tritordeum martini* A. Pujadas nothosp. nov.). Golden color of its flour-derived products is a distinctive quality criterion very appreciated by consumers. Hexaploid tritordeum is derived from the cross between the wild barley *Hordeum chilense* Roem. et Schultz. and durum wheat. Tritordeums exhibit a much higher yellow pigment content than their durum wheat parents (Alvarez, Martin, & Martin, 1999) due to the synthesis of higher amounts of carotenoids in the grains (Rodríguez-Suárez, Giménez, & Atienza, 2010). Carotenogenic genes from *H. chilense* contribute decisively to the seed carotenoid content of tritordeum. The up-regulation of *Phytoene synthase 1 (Psy1)* and *Lycopene epsilon-cyclase (Lcye)* in tritordeum is associated with the differences in seed carotenoid content between tritordeum and durum wheat (Rodríguez-Suárez, Mellado-Ortega, Hornero-Méndez, & Atienza, 2014). Besides, the *H. chilense* genome also modifies the carotenoid profile. Indeed, a significant proportion of the carotenoids at harvest are esterified with fatty acids in tritordeum (Atienza, Ballesteros, Martin, & Hornero-Mendez, 2007) and *H. chilense* (Avila, Mattera, Rodríguez-Suárez, Palomino, Ramírez, Martin et al., 2019; Mellado-Ortega & Hornero-Méndez, 2015). On the contrary durum wheat does not usually produce lutein esters (Ahmad, Mather, Law, Li, Yousif, Chalmers et al., 2015; Ziegler, Wahl, Würschum, Longin, Carle, & Schweiggert, 2015) although small amounts of lutein monoesters have been reported in this species (Atienza et al., 2007).

The transference of chromosome 7H^{ch} from *H. chilense* to wheat background increases the total carotenoid content (Mattera, Cabrera, Hornero-Méndez, & Atienza, 2015; Rey, Calderón, Rodrigo, Zacarías, Alós, & Prieto, 2015) likely due to the presence of *Psy1* in this chromosome (Rodríguez-Suárez et al., 2010). Besides, the simultaneous presence of chromosome 7H^{ch} and 7D in wheat-*H. chilense* substitution lines increases the amount and proportion of lutein esters along with the total carotenoid content (Mattera et al., 2015).

These findings are important for two main reasons. In first place, lutein esters are more stable than free lutein during post-harvest storage (Ahmad, Asenstorfer, Soriano, & Mares, 2013; Mellado-Ortega, Atienza, & Hornero-Méndez, 2015; Mellado-Ortega & Hornero-Méndez, 2016). Accordingly, breeding for higher carotenoid esterification may allow for higher lutein retention through the food chain. This is particularly important considering the high lutein losses reported during flour storage (Hidalgo & Brandolini, 2008) and during bread and pasta preparation (Hidalgo, Brandolini, & Pompei, 2010).

In second place, carotenoid esterification could be an important mechanism for carotenoid accumulation during grain development in tritordeum. Esterification is a common means of carotenoids sequestration in plants, as reported in pepper (Berry, Rickett, Baxter, Enfissi, & Fraser, 2019). The extended occurrence of xanthophyll esters in food from plant and animal origin has been recently reviewed (Hornero-Mendez, 2019). The generation of carotenoid sequestering structures and the improvement of plastid sink capacity to store carotenoids is considered a viable strategy to increase carotenoid content in plants (Rodríguez-Concepción et al., 2018). This sequestration is considered a regulatory mechanism providing a metabolic sink for carotenoid accumulation (Cazzonelli & Pogson, 2010). Ariizumi, Kishimoto, Kakami, Maoka, Hirakawa, Suzuki et al. (2014) showed that the formation of xanthophyll esters in tomato increases the amount of xanthophylls in the petals. They suggested that esterification increases xanthophyll sequestration and/or prevents the xanthophyll degradation. Similarly the overexpression of the dominant mutation *Orange* (*OR*) from cauliflower in rice allowed increasing the carotenoid accumulation in part by up-regulating endogenous carotenogenic genes (Bai, Capell, Berman, Medina, Sandmann, Christou et al., 2015). Similar findings were observed in tritordeum where carotenogenic genes from *H. chilense* were up regulated during grain development (Rodríguez-Suárez et al., 2014). Whether this up-regulation is caused by the synthesis of lutein esters (causing a metabolic sink) remains an open question.

The present work aims to investigate the existence of a metabolic sink during grain development in tritordeum and wheat-*H. chilense* genetic stocks that may contribute to the higher lutein accumulation promoted by the H^{ch} genome, and to determine the possible involvement of lutein esterification in this process.

2. Materials and Methods

2.1. Plant materials and experimental details

The plant materials used in this work included the common wheat genotype 'Chinese Spring' (CS); three CS-*H. chilense* chromosome substitution lines [CS 7H^{ch}(7A), CS 7H^{ch}(7B), CS 7H^{ch}(7D)] developed at John Innes Center, Norwich, UK (T.E. Miller and S.M. Reader, unpublished results; www.jic.ac.uk/germplasm/Wheat-Precise-Genetic_stocks-Aliens.pdf), and three hexaploid tritordeum genotypes, HT609, HT621 (Ballesteros, Ramirez, Martinez, Atienza, & Martin, 2005) and HT630, developed at Institute for Sustainable Agriculture – CSIC (Córdoba, Spain). All genotypes were sown in a field experiment following a completely randomized design with two replicates. Spikes were labelled indicating the anthesis date and harvested at different times (20, 24, 28, 32, 36 and 40 days post anthesis, dpa), frozen in liquid nitrogen and stored at -80 °C until carotenoid extraction. From each plot two samples consisting of several spikes were collected and analyzed independently.

2.2. Pigment analysis

Grain samples for each developmental stage were freeze-dried (lyophilized) to obtain both dry matter content (%) and moisture content (%). Subsequently, dry grains were milled using an oscillating ball mill Retsch Model MM400 (Retsch, Haan, Germany) fitted with a 25-mL stainless steel grinding jar together with two stainless steel balls (15 mm Ø) at 25 Hz for 1 min. Pigments

from developing grains at 20, 24, 28 and 32 dpa were extracted by using a one-step grinding-extraction procedure (Rodríguez-Suárez et al., 2014). Briefly, 0.2 g of flour (exactly weighted) were placed into 2-mL safe-lock round-bottom Eppendorf tube together with two stainless-steel balls (5 mm $\varnothing$) and 1 mL of acetone containing 0.1 % (w/v) BHT. The tubes were placed in an oscillating ball mill Retsch Model MM400 (Retsch, Haan, Germany) at 25 Hz for 1 min. Samples were centrifuged at 13,000 $\times g$ for 5 min at 4 °C, and the supernatant was directly used for HPLC analysis. At 36 and 40 dpa, due to the filling process in grains, carotenoid extracts need to be prepared with a higher sample size (1 g) preventing the use of one-step grinding-extraction procedure. Therefore, carotenoids from grains at 36 and 40 dpa were prepared according to Atienza et al. (2007).. Thus, one-gram of milled grain sample was placed in a round-capped polypropylene 15 mL centrifuge tube, and the pigments were extracted with 4 mL of acetone (containing 0.1 % BHT) during 2 min by vortexing, following sonication for 1 min. The mixture was centrifuged at 4,500 $\times g$ for 5 min at 4 °C, and the extracted carotenoids in the acetone phase were collected. The extraction operation was repeated three times, and the acetone fractions were pooled. The solvent was evaporated under nitrogen stream, and the pigments were dissolved in 0.5 mL of acetone and stored at -30 °C until HPLC analysis. Samples were centrifuged at 13,000 $\times g$ for 5 min at 4 °C prior to chromatographic analyses, which were carried out in the same day of the preparation of the extracts. All operations were performed under dimmed light to prevent isomerization and photodegradation of carotenoids.

2.3. Pigment identification

Identification of carotenoid pigments in all genotypes has been described in previous works (Atienza et al., 2007; Mellado-Ortega & Hornero-Mendez, 2012; Rodríguez-Suárez et al., 2014).

2.4. HPLC analysis of carotenoids

HPLC quantitative analysis of pigments was carried out according to the method of Mínguez-Mosquera and Hornero-Méndez (1993) with some modifications (Atienza et al., 2007). 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 C18 reversed-phase analytical column (Mediterranea SEA18, 3 μ m, 20 $\times$ 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 % deionised water. The gradient profile used for the chromatographic analysis of early developing grains (20, 24, 28 and 32 dpa) was increased linearly to 95 % acetone in 10 min, hold for 7 min, then raised to 100 % in 3 min, and maintained constant for 5 min. For developing grains at the late stages (36 and 40 dpa) the last stage of the gradient profile (100% acetone) was maintained constant for 13 min.

2.5. Statistical analyses

Analyses of variance for total carotenoid content and concentration of each compound were performed using Statistix version 10.0 (Analytical Software, Tallahassee, FL, USA) and IBM SPSS Statistics version 26. Differences between developmental stages or genotypes were established using Tukey's honest significance (HSD) test at $P < 0.05$.

3. Results and discussion

The typical pigment composition associated to chloroplast of green plant tissues was found in the six developmental stages analyzed. The pigment profile included several carotenoids along with chlorophylls *a* and *b*. The carotenoid fraction consisted of (9 $'$ Z)-neoxanthin, violaxanthin, (all-*E*)-antheraxanthin, (all-*E*)-zeaxanthin, (all-*E*)- β -carotene and lutein, the last one being the major carotenoid in all stages (majorly including the (all-*E*) isomer accompanied by small amounts of (9Z)- and (13Z)- isomers)]. For simplicity, carotenoids are referred hereinafter as lutein, β -carotene, neoxanthin, violaxanthin, antheraxanthin and zeaxanthin. Complete pigment composition for each genotype and developmental stages are shown in Online Resource 1.

For total carotenoid content, a general decreasing trend was observed in all the genotypes during grain development (Fig. 1). This trend can be mostly explained by both the increase in grain size (filling), which has a dilution effect, and the decrease on photosynthesis rate in the grain which decreased pigment turn-over and lead to pigment catabolism.

The chlorophyll content followed the same trend in all the samples with very low levels of both chlorophyll *a* and chlorophyll *b* at 32 days post anthesis (dpa), indicating the progressive loss of photosynthetic capacity and the disintegration of chloroplasts as grains developed (Online Resource 2).

Lutein is the main carotenoid at harvest in wheat and related species (Ficco et al., 2014; Lachman, Martinek, Kotíková, Orsák, & Šulc, 2017), usually representing over 90% of total carotenoids. The relative proportion of lutein in relation to the total carotenoid content was calculated for each genotype and grain developmental stage (Fig. 2). The relative importance of lutein in the carotenoid pool increased during grain development. The proportion of lutein was around 30-40% in CS and CS-*H. chilense* genetic stocks at 20 dpa but it represented around 50% of total carotenoid content in tritordeum which reflects the higher synthesis capacity of this species. From 20 dpa onwards, the proportion of lutein increased until 40 dpa where it

represented around 70% of total carotenoid in CS; 80% in CS-*H. chilense* genetic stocks and 90% in tritordeums. Carotenoids levels are determined by biosynthesis but also by degradation of carotenoids and storage/sequestration capabilities (Ruiz-Sola & Rodríguez-Concepción, 2012). The continued synthesis of lutein during grain development is supported by the up-regulation of *Psy1* and *e-Lcy* in tritordeum (Rodríguez-Suárez et al., 2014) and the expression of *Psy1* genes in durum wheat (Vargas, Schulthess, Royo, Matus, & Schwember, 2016).

To further investigate the differences in carotenoid accumulation during grain development between the genotypes studied in this work, we compared the total carotenoid content of each genotype with CS which is characterized by a low amount of total carotenoids at harvest (Mattera et al. 2015). Wheat-*H. chilense* genetic stocks share the CS background but they differ in the homoeologous group 7 chromosomes. All of them carry the chromosome 7H^{ch} which increases the carotenoid content in common wheat either as chromosome addition (Alvarez, Martin, & Martin, 1998), substitution or introgression lines (Mattera et al., 2015; Rey et al., 2015). The genetic stocks [CS (7A)7H^{ch}, CS (7B)7H^{ch} and CS (7D)7H^{ch}] did not differ from CS at 20 and 24 dpa but they showed a steadily increase until 40 dpa when they reached a total carotenoid content of 4-fold compared to CS (Fig. 1).

Tritordeums are characterized by a higher amount of carotenoids than the genetic stocks used in this work (Mattera et al. 2015) and wheat (Paznocht, Kotíková, Šulc, Lachman, Orsák, Eliášová et al., 2018). Our results show that tritordeums have a higher carotenoid content than CS at 20 dpa (1.5 fold) while the genetic stocks only differed from CS from 28-32 dpa onwards. Tritordeums have all the genes of the carotenoid pathway from *H. chilense* while the chromosome substitution lines only have *Psy1* from *H. chilense*. This difference explains the earlier and higher synthesis of tritordeum compared to the genetic stocks.

Since esterification is a common means for sequestering and accumulating carotenoids within the plant cells (Rodríguez-Concepción et al., 2018) we wondered whether the activation of the carotenoid pathway described by Rodríguez-Suárez et al. (2014) was caused, at least in part, by the early formation of lutein esters which would be acting as a metabolic sink and would result in higher lutein accumulation.

To test this hypothesis we investigated the carotenoid esterification profile during grain development (Fig. 3). No carotenoid esters were detected before 36 dpa despite both the genetic stocks and tritordeums increased their total carotenoid content relative to that of CS at earlier stages during grain development (Fig. 1). In addition, at 36 dpa lutein esters were only found in CS-7Hch(7A) and CS-7Hch(7B) and they were not detected in tritordeum despite their higher relative total carotenoid content (Fig. 3). The absence of lutein esters before 36 dpa suggests that esterification is not acting as a metabolic sink before this stage and thus it is not the main determinant of the up-regulation of the carotenoid pathway reported in tritordeum (Rodríguez-Suárez et al., 2014). After this stage (36 dpa) esterification may have a complementary role to contribute to the total carotenoid content in accessions producing esters by limiting carotenoid catabolism and subsequent losses, which is important to increase the retention of carotenoid in the food chain.

Low relative humidity promotes lutein esterification (Ahmad et al., 2013; Kaneko, Nagamine, & Yamada, 1995). Our results are in agreement with these findings since lutein esters were not detected until 36 dpa when seeds were already under the dehydration process. Lutein esters are more stable than free carotenoids *in vivo* (Mattera, Hornero-Méndez, & Atienza, 2017), which is in agreement with the higher stability described for carotenoid esters (Rodríguez-Concepción et al., 2018) and thus esterification may provide new opportunities for improving cereal quality by increasing the stability and retention of these pigments

Diversity for xanthophyll esterification has been reported in wheat including varieties such as 'Bohemia' (Paznocht, Kotíková, Orsák, Lachman, & Martinek, 2019) or breeding lines such as V1-131-15 (Paznocht et al., 2018) where only free lutein was detected. Similar findings for esterification variability have been reported in *H. chilense* (Avila et al., 2019). In this context, the higher amount of lutein esters of HT630 compared with HT621 and HT609 (Fig. 3) may indicate the existence of more efficient alleles or complementary genes for lutein esterification in HT630. Candidate genes for lutein esterification have been identified in chromosome 7D of wheat (Ahmad et al., 2015; Avila, Palomino, Hornero-Mendez, & Atienza, 2019b). Considering the high degree of synteny between *H. chilense* and other Triticeae species (Avila et al., 2019), the candidate genes identified in chromosome 7D should be investigated in this wild barley since they may be responsible for the esterification depending on the presence of chromosome 7H^{ch} observed in this and previous works (Mattera et al., 2015).

4. Conclusions

The lack of lutein esters before 36 dpa suggests that the up-regulation of the carotenoid pathway observed in early stages of grain development in tritordeum is not caused by the formation of a metabolic sink mediated by esterification. Nevertheless, a complementary role of lutein esterification in carotenoid accumulation cannot be ruled out since it may contribute to a lower carotenoid degradation rate at later stages. Differences in lutein esterification among tritordeums suggest the existence of diversity for xanthophyll acyl transferases which could be exploited for improving the nutritional value of tritordeum by increasing the stability and retention of lutein through the food chain.

Conflict of interest

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

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278 **Acknowledgements**

279 This research was funded by Grants AGL2014-53195-R from Ministerio de Economía y
280 Competitividad and AGL2017-85368-P from Ministerio de Ciencia, Innovación y Universidades.
281 Both projects are cofounded by FEDER. DHM is member of CaRed Network funded by Ministerio
282 de Economía y Competitividad (BIO2015-71703-REDT and BIO2017-90877-REDT) and
283 participates in EUROCAROTEN COST Action (CA15136). SGA is member of FiRCMe Network,
284 funded by Ministerio de Economía, Industria y Competitividad (AGL2016-81855-REDT). M.G.M.
285 was recipient of a fellowship from MINECO (BES-2012-055961).

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Caption figures

Fig. 1. Total carotenoids production during grain development. At each developmental stage (indicated as days post anthesis, dpa), genotypes with the same letter are no significantly different for total carotenoid content at $P < 0.05$, determined by Tukey's HSD test.

Fig. 2. Relative contributions of individual carotenoids (%) to the carotenoid pool during grain development. For each genotype, developmental stages (indicated as days post anthesis, dpa) with the same letter are not significantly different for the relative contribution of lutein (%) at $P < 0.05$, determined by Tukey's HSD test.

Fig. 3. A. Accumulation of lutein esters during grain development. B. HPLC chromatogram corresponding to the carotenoid profile of developing grains of tritordeum at 40 dpa. Peak identities: 1, (all-E)-lutein; 2, (9Z)-lutein; 3, (13Z)-lutein; 4, (all-E)- β -carotene; ME, lutein monoesters; DE, lutein diesters.

Supplementary file 1. Detailed analysis of the pigment composition including carotenoid esters and chlorophyll.

Supplementary file 2. Chlorophyll trends during grain development.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

None

Tritordeum has a higher carotenoid content than wheat from 20 days post anthesis (dpa)
Lutein esters were not detected before 36 dpa

475 Lutein esters do not produce a metabolic sink during early stages of grain development
476 Differences among tritordeums for lutein esters suggest diversity for xanthophyll acyl-
477 transferase (XAT) enzymes
478
479
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