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Carotenoid profiling of *Hordeum chilense* grains: The parental proof for the origin of the high carotenoid content and esterification pattern of tritordeum

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ABSTRACT

The outstanding high carotenoid content of the tritordeum (×*Tritordeum* Ascherson et Graebner) grains, a promising novel cereal derived from the crossing of durum wheat and the wild barley *Hordeum chilense*, has previously been assigned as a character derived from the genetic background of its wild parent. The carotenoid profile of *H. chilense*, especially the lutein esters presented in this study, provide biochemical evidences to confirm this affirmation, being the first time that the individual carotenoid profile of this cereal has been characterized. The total carotenoid content (6.14 ± 0.12 μg/g) and the individual carotenoid composition were very similar to the tritordeum grains, with lutein being the major carotenoid (88%; 5.38 ± 0.11 μg/g) and very low levels of β-carotene. In contrast to tritordeum, *H. chilense* presented a considerable amount of zeaxanthin (12%; 0.74 ± 0.01 μg/g). Up to 55% of lutein was esterified with palmitic (C16:0) and linoleic (C18:2) acids, presenting a characteristic acylation pattern, in agreement with the tritordeum one, and composed by four monoesters (lutein 3′-*O*-linoleate, lutein 3-*O*-linoleate, lutein 3′-*O*-palmitate and lutein 3-*O*-palmitate) and four diesters (lutein dilinoleate, lutein 3′-*O*-linoleate-3-*O*-palmitate, lutein 3′-*O*-palmitate-3-*O*-linoleate, lutein dipalmitate). These data may be useful in the field of carotenoid biofortification of cereals.

1. Introduction

Carotenoid pigments are lipophilic molecules responsible for the red, orange and yellow colors of most fruits and vegetables and certain animals. The latter, including humans, are unable to synthesize carotenoids *de novo*, so they need to incorporate them in the diet. Carotenoids play their basic functions as light collectors in the photosynthetic apparatus of plants, besides preventing oxidative damage as potent antioxidants. As a result of the antioxidant property, important functions for human health are derived, such as the prevention of certain degenerative and chronic diseases (Fernández-García et al., 2012). Although the cereal grains have a relatively low carotenoid content compared to most fruits and vegetables, the daily intake of cereals and cereal derived products by the majority of the population, makes these staple food a non-negligible and affordable source for carotenoids and other phytochemicals (Graham and Rosser, 2000), becoming ideal vehicles to be used in biofortification and nutritional strategies (Bai et al., 2011).

Since the beginning of the twentieth century, an increasing interest has been raised among cereal breeders for the development of interspecific hybrids in order to obtain new cereals with increased phytochemical contents and improved agronomic performance and technological qualities. One of the success stories in cereal breeding is the generation of tritordeum (×*Tritordeum* Ascherson et Graebner), the fertile amphiploids derived from the crossing of durum wheat and a wild barley (*Hordeum chilense* Roem. & Schult.) (Martín and Sanchez-Monge Laguna, 1982; Martín et al., 1999). *H. chilense* is a wild diploid barley ($2n = 2x = 14$) belonging to the section *Anisolepsis Nevski*, being an extremely variable species included in a heterogeneous group of South American species of the genus *Hordeum*, carrying the H genome. The use of this wild cereal in breeding programs has focused on two main areas; first, the development of *Tritordeum* amphiploids between *H. chilense* and tetraploid (*Triticum turgidum* Desf.) or hexaploid (*Triticum aestivum* L.) wheats with the aim of obtaining a

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new functional cereal; and second, the introgression to wheat of new traits of interest (agronomical, technological, nutritional, etc). One of the main interests of this species is its potential for increasing the carotenoid content in durum wheat (Rodríguez-Suárez et al., 2011). The color of durum wheat semolina is mainly due to the carotenoid pigments of the grains, being considered an important quality criterion with regard to pasta production (Hentschel et al., 2002). This quality trait has been frequently assessed as YPC (yellow pigment content). The genetic variation of endosperm color trait in tritordeum and its relationship to the level of pigments in both parental species, *H. chilense* and durum wheat has also been characterized (Álvarez et al., 1999).

The small grains of *H. chilense* are characterized by a high level of carotenoids, presenting at least two loci (QTLs) for the pigment content trait located on the $2H^{ch}$ and $7H^{ch}$ chromosomes, showing a high genetic variability for this trait (Álvarez et al., 1998, 1999; Atienza et al., 2004). More recently, twelve genes related to endosperm carotenoid content in grasses (*Dxr*, *Hdr*, *Ggpps1*, *Psy2*, *Psy3*, *Pds*, *Zds*, *e-Lcy*, *b*-Lcy, *Hyd3*, *Ccd1* and *Ppo1*) have been mapped in *H. chilense*, and additionally a new main region associated with YPC has been identified in $3H^{ch}$ chromosome (Rodríguez-Suárez and Atienza, 2012). These findings provide the first steps towards the implementation of a MAS (Marker Assisted Selection) program for identifying genes determining a higher carotenoid in *Tritordeum*, and for development of new tools and strategies for transferring these genes and traits from selected amphiploids to wheat lines under breeding programs (Atienza et al., 2007a). In previous works, it has been demonstrated that the advanced tritordeum lines showed carotenoid levels up to 8-times higher than their parental durum wheat cultivars, with lutein being the major pigment (Atienza et al., 2007b; Mellado-Ortega and Hornero-Méndez, 2012). Moreover, a high proportion of lutein (up to 40%) in tritordeum is presented as mono- and diesters (homo- and heterodiesters) with a specific set of two fatty acids, palmitic and linoleic acids, for which different regioisomers of monoesters and heterodiesters have been identified and characterized in tritordeum for the first time in a cereal grain (Mellado-Ortega and Hornero-Méndez, 2012). There are experimental evidences suggesting that the esterification of xanthophylls is an important mechanism and strategy in vegetables for sequestering and accumulating these lipophilic compounds within the plastids (Fernández-Orozco et al., 2013; Hornero-Méndez and Mínguez-Mosquera, 2000). According to previous reports, the esterification of xanthophylls, such as lutein and β-cryptoxanthin, with fatty acid increased their stability against heat and light (Fu et al., 2010; Subagio et al., 1999), and preserved the antioxidant activity similar to the free carotenoid (Subagio and Morita, 2001). Therefore the correct understanding of the biochemical pathway governing the esterification of xanthophylls seems to be crucial in order to implement strategies for increasing the carotenoid content of crops. In this way, we have proposed tritordeum grains as an excellent plant model for deciphering the functions and significance of the esterification of the xanthophyll process in plants, including the characterization of the xanthophyll acyltransferase enzymes (XAT) and the acyl donor molecules (acyl lipids and/or free fatty acids) involved in this still unknown pathway (Mellado-Ortega and Hornero-Méndez, 2012).

As far as we know to date, only one of the parental cereal species of the amphiploid tritordeum, durum wheat, has been fully characterized in relation to the individual carotenoid composition (Abdel-Aal et al., 2007; Atienza et al., 2007b; Blanco et al., 2011; Hentschel et al., 2002). Therefore, the present study was aimed at characterizing the carotenoid profile in grains of *H. chilense*, with the aim of expanding knowledge about the carotenogenic process in tritordeum, as well as to provide biochemical evidences to support the hypothesis that the origin of the esterification pattern of tritordeum is a character mostly derived from the genetic background of this parental (*H. chilense*).

2. Materials and methods

2.1. Plant material

Grains of *H. chilense* (ascension PI 531781 D-2739) were obtained from the National Small Grains Collection (NSGC) of the National Plant Germplasm System (NPGS) of the United States Department of Agriculture – Agricultural Research Service (USDA-ARS). For comparative purposes, grains of a commercial variety of durum wheat (*T. turgidum*, Don Pedro cultivar) and an advanced line of tritordeum (HT621) were also analyzed. HT621 was developed within the Cereal Breeding Program of the Institute for Sustainable Agriculture (IAS-CSIC, Córdoba, Spain) and is deposited at the USDA National Plant Germplasm System (ref. PI 636334), being characterized for presenting a high carotenoid content.

2.2. Chemicals and reagents

HPLC-grade methanol, methyl tert-butyl ether (MTBE) and acetone were supplied by BDH Prolabo (VWR International Eurolab, S.L., Barcelona, Spain). HPLC-grade deionized water was produced with a Milli-Q 50 system (Millipore Iberica S.A., Madrid, Spain). The rest of reagents were all of analytical grade.

2.3. Extraction of carotenoids

Carotenoid pigments were extracted from tritordeum and durum wheat grains according to Mellado-Ortega and Hornero-Méndez (2012). In the case of *H. chilense*, due to the limiting available material, some modifications were introduced in order to down-scale the procedure. Briefly, the plant material (0.15 g; ca. 50 seeds) was ground with a ball mill (MM400 Retsch) by placing the seeds in a 2 mL safe-lock Eppendorf® tube together with two stainless-steel balls (5 mm diameter) during 1 min at 25 Hz rate. Carotenoids were subsequently extracted with 1 mL of acetone (containing 0.1% BHT), centrifuged at 13,500 × *g* for 5 min at 4 °C, and the supernatant was directly used for the chromatographic analysis. Only a one-step solvent treatment was necessary for the complete extraction of pigments (data not shown). All operations were carried out under dimmed light to prevent isomerization and photodegradation of carotenoids. Analyses were carried out in quadruplicate.

2.4. Pigment identification

The procedure for the identification of carotenoid pigments and their esters in *H. chilense* was the same as already described in previous works for durum wheat and tritordeum (Atienza et al., 2007b; Mellado-Ortega and Hornero-Méndez, 2012). However, due to the limitation of the available plant material, the identification of carotenoid pigments was mainly based on the chromatographic (retention time) and spectroscopic (UV–visible and MS) properties obtained by HPLC–DAD and HPLC–DAD–MS(APCI+), as well as some micro-scale chemical tests for the determination of the presence 5,6-epoxide, hydroxyl and carbonyl groups.

As described by Mellado-Ortega and Hornero-Méndez (2012), the structural assignment of the lutein esters, including the regioisomers, was mainly based on the fragmentation pattern obtained under the liquid chromatography mass spectrometry (LC-MS (APCI+)) conditions described below. Moreover, the tentative identification of *cis* isomers of lutein was based on the presence and