Hordeum chilense genome, a useful tool to investigate the endosperm yellow pigment content in the Triticeae

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Abstract

Background: The wild barley Hordeum chilense fulfills some requirements for being a useful tool to investigate the endosperm yellow pigment content (YPC) in the Triticeae including its diploid constitution, the availability of genetic resources (addition and deletion stocks and a high density genetic map) and, especially, its high seed YPC not silenced in tritordeums (amphiploids derived from H. chilense and wheat). Thus, the aim of this work was to test the utility of the H. chilense genome for investigating the YPC in the Triticeae.

Results: Twelve genes related to endosperm carotenoid content and/or YPC in grasses (Dxr, Hdr [synonym ispH], Ggpps1, Psy2, Psy3, Pds, Zds, e-Lcy, b-Lcy, Hyd3, Ccd1 and Ppo1) were identified, and mapped in H. chilense using rice genes to identify orthologs from barley, wheat, sorghum and maize. Macrocolinearity studies revealed that gene positions were in agreement in H. vulgare and H. chilense. Additionally, three main regions associated with YPC were identified in chromosomes 2Hch, 3Hch and 7Hch in H. chilense, the former being the most significant one.

Conclusions: The results obtained are consistent with previous findings in wheat and suggest that Ggpps1, Zds and Hyd3 on chromosome 2Hch may be considered candidate genes in wheat for further studies in YPC improvement. Considering the syntenic location of carotenoid genes in H. chilense, we have concluded that the Hch genome may constitute a valuable tool for YPC studies in the Triticeae.

Keywords: Yellow pigment content (YPC), Macrocolinearity, Candidate genes, H. chilense, H. vulgare

Background

Hordeum chilense Roem. et Schultz. is a diploid wild barley included in the section Anisolepis Nevski, native to Chile and Argentina. This species has been used as a new source of cytoplasmic male sterility for hybrid wheat production [1-3] and it shows other potentially useful traits for wheat breeding, including immunity to Septoria tritici blotch and high seed yellow pigment content (YPC) [4-6]. The compatibility of the H. chilense genome with Triticum spp. is high, and fertile amphiploids (×Tritordeum Ascherson et Graebner), have been developed from the hybrid between H. chilense and durum or common wheat [7,8]. Tritordeums are subjected to a breeding program at IAS-CSIC conducted by Prof. A. Martín with the goal of obtaining a new crop. Additionally, they are also useful as a genetic bridge to transfer H. chilense genes to both common and durum wheat [9].

One of the most valuable traits of this new cereal is the high endosperm YPC given by H. chilense genes. The YPC, mainly caused by carotenoids, is also very important in durum wheat due to its relation to semolina quality [10-13]. In addition, the consumption of carotenoid-rich foods has been associated with a reduced risk of developing certain types of diseases [14,15]. Besides, carotenoids with unsubstituted β-ionone rings, such as β-carotene, have provitamin A activity [16] while other carotenoids such as lutein may prevent age-related macular degeneration [17,18]. Accordingly, seed carotenoid content is receiving more attention with the goal of developing new functional foods.

The carotenoid biosynthesis pathway is well known in plants [16,19-22]. Candidate gene studies in maize have shown the role of several carotenoid-related genes in...
endosperm carotenoid content using quantitative trait locus (QTL) and association studies. Indeed, several genes including *Psy1* [23-25]; *Zds* [25]; e-
*Ley* [26]; *Ziso* [27] and *Hyd3* [28] have been related to endosperm carotenoid content. Genes from both upstream and downstream pathways have also been investigated at the transcriptomic level in maize [29].

Despite the importance of YPC in durum wheat and tritordeums and the positive correlation between YPC and carotenoid content, candidate gene studies for YPC in both species are mostly based on *Phytoene synthase 1* gene (*Psy1*). *Psy1* catalyzes the first step of the carotenoid biosynthetic pathway and it is considered a limiting factor for carotenoid production [18,30]. Since the first reports about the location of *Psy1* to chromosomes 7A and 7B in wheat [31,32], a considerable amount of work has demonstrated the role of this gene in durum wheat YPC [33-39]. In addition, the lycopene *epsilon cyclase* gene (*e-Ley*) has been co-localized with a QTL for YPC in wheat [35]. Nevertheless the role of other candidate genes for YPC in durum wheat remains largely unexplored. Given the high level of macrocolinearity among grasses, the knowledge gained in any species may be useful in others. This is more evident within the *Triticaceae* due to the close relationship among these species. Indeed, *Triticaceae* species are represented as a single genome in comparative studies [40,41].

In this context *H. chilense* may be a useful species to investigate the role of carotenoid-related genes in the YPC in the *Triticaceae*. First, it exhibits a high YPC which is not silenced in wheat background, as evidenced in tritordeums [10,42,43]. Second, the diploid constitution of this species and the existence of *H. chilense*-wheat chromosome addition lines [44] and other deletion stocks [45,46] makes the identification of candidate genes more accessible. Third, a high density genetic map has been recently developed using *H. chilense*-derived DArT markers [47] which allows efficient candidate gene mapping and comparative studies. The objective of this work is to determine whether *H. chilense* could be useful for investigating the endosperm YPC in the *Triticaceae*. For this purpose we aimed to identify and locate a set of carotenoid-related genes previously studied in other grasses and to determine the macrocolinearity between *H. chilense* and barley for these genes. In the second place, we aimed to identify the main regions for YPC in *H. chilense* and to discuss the results obtained in relation to wheat.

**Methods**

**Plant material**

*Hordeum chilense* accession lines H1 and H7 from the collection of the Instituto de Agricultura Sostenible (IAS-CSIC, Córdoba) were used for polymorphism detection in the selected genes. DNA of 92 F7-Recombinant Inbred Lines (RILs) derived from the cross H1 × H7 was used for genetic mapping of the gene-based markers designed. These lines were sown in small pods with five seeds each. After initial growing, RILs were transplanted to field conditions using a randomized plot design with two replications.

For physical mapping, the following common wheat cv. Chinese Spring (CS)-*H. chilense* addition lines were used: with complete chromosomes 1Hch, 4Hch, 5Hch, 6Hch or 7Hch (named CS MA 1Hch-1 HchS, CS DA4Hch, CS DA5Hch, CS DA6Hch, and CS DA7Hch, respectively, where MA refers to monosomic addition and DA to disomic addition), and (CS)-*H. chilense* ditelosomic addition lines CS DA1HchS, CS DA2HchS, CS DA5HchL, CS DA6HchS, CS DA7HchS, CS DA7HchA, CS DA7HchB.

Leaf tissue was harvested at tillering stage, frozen in liquid nitrogen, and stored at -80°C. Genomic DNA was extracted using the CTAB method according to [48].

**Primer design**

The twelve genes were selected from the main steps of the carotenoid biosynthetic pathway but also from upstream and downstream pathways, considering previous results obtained in other grasses, mainly maize. Rice genes were used as a query to identify orthologous genes in maize, sorghum, wheat and/or barley (see Additional file 1). Nucleotide sequences were aligned to design primers in the conserved regions of each gene. *Psy1* sequence of *H. chilense* was available from previous works [31,49]. Sequences were aligned using Edalign program (http://emboss.sourceforge.net/index.html) and edited using GeneDoc software (http://www.psc.edu/biomed/genedoc). Sequence identity searches were performed at the NCBI (http://www.ncbi.nlm.nih.gov) using BLAST. Primer pairs were designed using Primer3plus software [50] on exonic regions flanking at least one intron when possible.

**PCR amplification and polymorphism detection**

PCR amplifications were performed in 25 μl reactions consisting of 0.625 units of DNA polymerase (Biotools B&M Labs, Madrid, Spain), 1× PCR buffer, 1.6 mM MgCl2, 320 mM dNTPs (Promega, Madison, WI, USA), 0.6 mM of each primer and 50 ng of genomic DNA. PCR reactions were also performed: 5 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 58°C (54°C for *Zds*) and 1 min at 72°C followed by 7 min at 72°C. For allele-specific amplifications with tetra-primer PCR, the number of cycles was increased to 40 and the annealing temperature was set at 60°C. Amplification and digestion of *Psy1* gene were carried out as described by [31]. PCR products were resolved on 2% agarose gels, stained with

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**References**

[23-25]: Various studies on carotenoid metabolism in plants.

[25]: Important gene for carotenoid production in maize.

[31-49]: Previous works on carotenoid biosynthesis in grasses.

[48]: CTAB method for genomic DNA extraction.

[49]: Previous works on *Psy1* sequence in *H. chilense*.

[50]: Primer3plus software for primer design.
ethidium bromide and visualized under UV light. All primers used in this work are summarized in Table 1.

Nucleotide sequences were obtained by direct sequencing or by cloning if more than one product was observed in the amplifications. For cloning, pGEMT-Easy vector (Promega, Madison, WI) was used to transform competent *Escherichia coli* (DH5α) cells. Plasmids were isolated and purified using Illustra plasmid Prep Mini Spin Kit (GE Healthcare, UK Ltd, UK).

Digestion reactions with restriction enzymes *HhaI, HinfI, MboII, Nhel* and *TasI* (Takara Bio Inc., Japan) were carried out following manufacturer’s instructions using 10 μl of PCR in a final volume of 20 μl.

**Identification of genomic regions for endosperm yellow pigment content (YPC)**

YPC was determined according to the AACC method 14–50 [51] in 71 F7-RILs, which yielded a sufficient amount of seed. Briefly, approximately 150 mg of flour was used to evaluate YPC. Pigment extraction was performed using water-saturated n-butanol in a 1:10 (w/v) ratio. Samples were centrifuged at 14,000 g for 10 min and the pigment was measured by absorbance reading at 435.8 nm. All determinations were performed in duplicate.

A DArT-based *H. chilense* map [47] constituted the basis for mapping. A framework map composed by markers spaced 3–5 cM was considered to determine trait-marker associations and QTL mapping since the use of very proximal markers results in highly similar LOD scores while it signifies a huge increase in the number of calculations. Gene-based markers were integrated in the *H. chilense* map using JoinMap 4.0 mapping software [52]. After linkage analysis, the position of each candidate gene within a chromosome was determined using the fixed order option, the Monte Carlo Maximum Likelihood Mapping algorithm (ML-mapping) module and the Kosambi mapping function.

Associations between markers and YPC were determined using the Kruskal-Wallis (KW) test and MapQTL 4.0 [53]. Regions showing at least five markers associated with YPC at *p* < 0.01 were considered as YPC regions but further analyses were performed to identify the most significant ones. Marker cofactors were automatically selected using the automatic cofactor selection (ACS) tool at *p* < 0.05. For both Interval Mapping (IM) and Multiple QTL Mapping (MQM), the genome-wide significance threshold was estimated using the permutation test with the following parameters: the walk speed was set to 3 cM; the number of permutations was set to 500 and the significance level to 0.05. The QTL position was estimated at the peak LOD with a 2-LOD support interval [54]. The map figure included in the manuscript was produced using MapChart software [55].

## Results

**Gene-based marker design, amplification and mapping**

A total of twelve genes not previously mapped in *H. chilense* were selected for this work: 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*Dxr*); 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (*Hdr* [synonym *ispH*]) from the MEP pathway; geranylgeranyl diphosphate synthase (*Ggpps1*); phytoene synthase 2 (*Psy2*), phytoene synthase 3 (*Psy3*); phytoene dehydrogenase (*Pds*); *Zeta*-carotene desaturase (*Zds*), lycopene epsilon cyclase (*Lbc*)

### Table 1 Primers designed for amplification and mapping of the candidate genes

| Primer   | Sequence 5’-3’          | Primer   | Sequence 5’-3’          |
|----------|--------------------------|----------|--------------------------|
| GGPPS1-F | GCCACGCGTCGACTCTCTAC     | PSY3-F   | TCGACGAGCTGTACTCTCAGT    |
| GGPPS1-R | GGAAACAGCAACCAAATGAT     | PSY3-R   | CTGACTCTGCTCCAGGATCT    |
| GGPPS1-spF | CGTATGCTTCTTCTAAGAGTG | HYD3-F3  | CTGCCGTTGTACTCCGATCCA   |
| GGPPS1-spR | GGAGATACCTATGCAAATCAT  | HYD3-R2  | CGACTTGTCTCATGCGTGTTT   |
| DXR-F1   | GAGCATTGGGAGAGAAAAATCA   | ZDS-F2   | GTTCACCGGTAACAAAGAC     |
| DXR-R1   | GGTCGACCTCGGACGATAGA     | ZDS-R2   | AGCTTGAGTCGAGGACCTT     |
| HDR-F2   | GGCATGCAACCATCAACAC     | LBC-F2   | CAAGCTAAGTCACATGAG      |
| HDR-R2   | TTTCCCTGTGCAATCTTCCTTG  | LBC-R2   | TGTCGAAACCGACAGTAT      |
| LEC-F2   | CTGAGCAATATGCGCTGAGA     | LBC-spF  | GAGCTCCACCCCTCCCT      |
| LEC-R2   | GGAGGTGCTCTGAGAGGTTCC   | LBC-spR  | GATCGCGGAGGCTCCTG       |
| PDS-F1   | TACAGTCGTTGATGCTGTTG    | CCD-F1   | TTGTACGAAAGGAAGACGCT    |
| PDS-R1   | GGAAATCAAACGGCCTGTA     | CCD-R1   | CAGCTCATCCCCACGGAAGTCT  |
| PSY2-F   | ATTCGTCGCGAGCTCAAGG     | CCD-spR  | GAAGTTCTCAGCTGCTGCG     |
| PSY2-R   | TGGAGTCGTTGCTCCCTGAT    | PPO1-F1  | AGCTCGGAGCTAGCTG        |
| PSY2-spR | GAGTCAAACATGCTTGAATGA   | PPO1-R1  | GTCCGCGGAGAAGAC         |
(e-Lcy), lycopene beta cyclase (b-Lcy) and beta-carotene hydroxylase 3 (Hyd3) from the carotenoid biosynthetic pathway; the carotenoid cleavage dioxygenase 1 gene (Ccd1) mediating the degradation of carotenoids to apocarotenoids and polyphenol oxidase 1 gene (Ppo1) implicated in plant tissue enzymatic browning.

Rice genes (www.gramene.org) were used as a query to identify orthologs from barley, wheat, sorghum and maize. Exon-intron boundaries and highly conserved regions were recognized by the alignment of each sequence set. Primers were designed on conserved exonic regions to amplify at least one intron and maximize polymorphism detection. Amplifications with the primers designed (Table 1) were carried out using the parental lines H1 and H7 as template. PCR products were sequenced to confirm their identity and examined for polymorphisms by comparing both parental lines.

All the candidate genes were successfully amplified and sequenced in H. chilense [GenBank: JQ922078-JQ922105]. Differences between H1 and H7 sequences were found for all the candidate genes, these being in most cases Single Nucleotide Polymorphisms (SNP) (Table 2). These SNPs were used to develop either Cleaved Amplified Polymorphism (CAP) markers for (Table 2). These SNPs were used to develop either single nucleotide polymorphisms by comparing both parental lines.

Regarding Ppo1, two sequences were amplified in H1 with primer pair PPO1-F1/PPO1-R1 differing in two SNPs (JQ922103 and JQ922104), both of them having in common a deletion of 23 bp compared to H7 (JQ922105). This was the length polymorphism scored in the mapping population.

All gene-based markers were located in the H. chilense map (Figure 1) in the following chromosomes: 2H<sup>ch</sup> (Ggpps1, Zds, Hyd3 and Ppo1); 3H<sup>ch</sup> (Dxr and e-Lcy); 4H<sup>ch</sup> (Hdr and Pds); 5H<sup>ch</sup> (Ccd1, Psy2 and Psy3); 6H<sup>ch</sup> (b-Lcy). The position (cM) of each candidate gene in the map is shown in Table 3. Both Ggpps1 and Zds were tightly linked and mapped near the centromere which was estimated in a 11 cM window [47] while Hyd3 and Ppo1 were mapped to the long arm of chromosome 2H<sup>ch</sup> at 142.38 cM and 161.56 cM respectively. The e-Lcy gene was located in the vicinity of the chromosome 3H<sup>ch</sup> centromere while Dxr was mapped to the distal part of the 3H<sup>ch</sup>S arm (Figure 1). Hdr and Pds genes were mapped to 4H<sup>ch</sup>S and 4H<sup>ch</sup>L arms respectively. Ccd1 (distal) and Psy2 (39.06 cM) were mapped to chromosome 5H<sup>ch</sup>S while Psy3 was mapped to the long arm of this chromosome (133.75 cM). Finally, b-Lcy was mapped to the long arm of chromosome 6H<sup>ch</sup> (111.2 cM) since the centromere is estimated at 84.0 cM [47]. Also, Psy1 is located on chromosome arm 7H<sup>ch</sup> as shown in previous studies using physical mapping [31,47].

Physical mapping by using the series of addition lines of H. chilense in common wheat was also possible for genes Hdr, Ggpps1, Psy2, Zds and Ccd1. Physical location could not be confirmed for genes located on

| Gene  | Accession numbers | Polymorphism detection | Physical mapping |
|-------|-------------------|------------------------|-----------------|
|       |                   | Genetic mapping        | Physical mapping |
|       | H1                | H7                     |                 |
| Dxr   | JQ922078          | JQ922079               | Allele size     |                 |
| Hdr   | JQ922096          | JQ922097               | Mboll digestion | Mboll digestion |
| Ggpps1| JQ922098          | JQ922099               | Allele specific primers | Allele specific primers |
| Psy2  | JQ922084          | JQ922085               | Allele specific primers | Allele specific primers |
| Psy3  | JQ922082          | JQ922083               | Hhal digestion  | Hhal digestion  |
| Pds   | JQ922092          | JQ922093               |                 |                 |
| Zds   | JQ922090          | JQ922091               | Hfii digestion  | TaI digestion  |
| e-Lcy | JQ922094          | JQ922095               | Hhal digestion  |                 |
| b-Lcy | JQ922088          | JQ922089               | Allele specific primers |                 |
| Hyd3  | JQ922080          | JQ922081               | Nhel digestion  |                 |
| Ccd1  | JQ922086          | JQ922087               | Allele specific primers | Allele specific primers |
| Ppo1  | JQ922103          | JQ922105               | Allele size     |                 |

Polymorphism between H1 and H7 lines of H. chilense used in genetic and physical mapping of the candidate genes selected in this work. GeneBank accession number for each sequence is given. Psy1 is not included as sequencing and mapping have been reported elsewhere.
chromosomes 2H\textsuperscript{chL} and 3H\textsuperscript{ch} (since there are no addition lines for these chromosomes) or when polymorphism with wheat was not detected with the designed markers (b-Lcy, Psy3, Pds and Ppo1).

To study the macrocolinearity between *H. chilense* and barley for these candidate genes, the *Poaceae* orthologous group corresponding to each candidate gene was identified from [http://rice.plantbiology.msu.edu/annotation_pseudo_pog.shtml](http://rice.plantbiology.msu.edu/annotation_pseudo_pog.shtml) using the rice gene as query (Table 3). Orthologous genes in *Brachypodium* and *Sorghum* were identified using the Barley Genome Zipper [56], which allowed the determination of their relative positions in the barley map [57] in all cases except for *Zds* which was not found in the Barley Genome Zipper (Table 3). The *Psy1*, previously mapped in *H. chilense*, was also located in barley.

**Figure 1** Marker-YPC association in the Kruskal-Wallis (KW) analysis. To determine marker-YPC associations, a framework map composed by markers spaced around 3–5 cM was considered from the map developed by [47]. The estimated centromere position is shown as a coloured segment within each chromosome. In chromosomes 6H\textsuperscript{ch} and 7H\textsuperscript{ch} centromeres were located in a single position since markers belonging to different chromosome arms mapped by this analysis. For chromosomes 6H\textsuperscript{ch} and 7H\textsuperscript{ch}, the estimated centromere locations are shown as black triangles. The position of the candidate genes mapped in this work is highlighted using a coloured box. The significance of the KW analysis is shown: - Not significant; *0.1; **0.05; ***0.01; ****0.005; *****0.001; ******0.0005; *******0.001. Three locations with at least five markers associated with YPC were considered as YPC regions (Regions 1 to 3). The most significant zone within these regions was determined using MQM analysis and permutation test (identified as YPC at 2HchL) and its position estimated at the peak LOD with a 2-LOD support interval.

**Discussion**

**Macrocolinearity between *H. chilense* and *H. vulgare**

Comparative analysis of the twelve candidate genes positions in *H. chilense* and barley using the Barley Genome Zipper [56] revealed a good agreement between both species for the candidate genes selected (Table 3). Indeed, chromosome location and relative positions of genes within chromosomes were conserved in all cases. Regarding *Zds*, its position could not be established in barley since the orthologous genes from rice, sorghum or *Brachypodium* were not found in the Barley Genome Zipper [56]. However, the close linkage between *Zds* and *Ggpps1* in *H. chilense* suggests that the two genes are located in equivalent positions in barley. This is supported by the high-resolution comparative analysis...
between barley and rice performed by [56]. Briefly, the barley Genome Zipper establishes the putative linear order of 21,766 barley genes by integrating gene indices of rice, sorghum and Brachypodium [56] and allows the establishment of the synteny relations between barley and rice chromosomes based on the position of the putative orthologs in both species. Chromosome 2H in barley corresponds to chromosome 4R in rice, except for a region in 2HS arm which is equivalent to 7R. In barley the region near the 2H centromere shows significant matches to both 7RS (where Zds is located in rice) and 7RL (where Ggpps1 is located in rice). This is in agreement with *H. chilense* results and suggests that Zds would be located in an equivalent position in barley.

An apparent break in the synteny between *H. chilense* and *H. vulgare* happens for *Psy1* which is located in 7HL in barley and in 7H\textsuperscript{ch}α in *H. chilense* [31]. However, genetic mapping could not establish the relative position of *Psy1* in 7H\textsuperscript{ch} chromosome since it remained in a short linkage group not linked to 7H\textsuperscript{ch} chromosome. Thus, its putative location in 7H\textsuperscript{ch}α is based upon physical mapping using a telosomic addition line. We have recently observed a reorganization of the *H. chilense* chromatin in wheat background between different chromosomes (A. Martín, personal communication). Thus, a translocation involving *Psy1* and 7H\textsuperscript{ch}S during the development of the addition line cannot be totally excluded and further work is required to confirm the position of *Psy1* in the chromosome 7H\textsuperscript{ch}.

### YPC regions: comparative analysis between *H. chilense* and wheat

The association study for YPC allowed the identification of three main regions in chromosomes 2H\textsuperscript{ch}, 3H\textsuperscript{ch} and 7H\textsuperscript{ch} for YPC variation in *H. chilense* as revealed by a KW association test. Despite a single QTL at the distal part of chromosome 2H\textsuperscript{ch} being above the significance threshold established, all three regions were considered significant since the population size critically affects the power of QTL detection and the precision of QTL estimates [58]. For instance, QTLs with small effects for stripe rust resistance in barley were detected only by increasing the population size [59]. The significance threshold must thus be chosen according to the population size and the trait of interest [58]. Therefore, we relied on KW-defined regions and considered the QTL analyses to conclude that the distal part of chromosome 2H\textsuperscript{ch} was the most significant one. Seven of the candidate genes were not associated with YPC in this work. This may indicate that they do not contribute to YPC variation in this population. Alternatively, these genes might have a minor effect which cannot be detected with this population size.

The region located in chromosome 3H\textsuperscript{ch} includes the *e-Lcy* gene, whose role in YPC has been demonstrated in hexaploid wheat and maize. In wheat, *e-Lcy* co-localized with a QTL for lutein accumulation and a specific mutation causing lutein content variations was identified [35]. This QTL was located in the proximal 3BS region this being the position of this gene in hexaploid wheat in...
agreement with the results obtained in *H. chilense*. Similarly, it has been shown that a QTL for carotenoid accumulation in maize chromosome 8 co-locates with e-Ley gene [25,26].

The distal part of chromosome 7H<sup>ch</sup>L is also related to YPC in *H. chilense*. The importance of this region has been clearly demonstrated in wheat, as a result of variations in *Psy1* genes [32,33,38]. This has promoted extensive work towards the identification of *Psy1-A* and *Psy1-B* alleles, the determination of their role in YPC and the development of functional markers for selection [34,60,61]. Furthermore, there is evidence of an additional locus for YPC in the distal part of chromosome 7AL in durum wheat [38]. Given that *Psy1* is apparently located in the 7H<sup>Ch</sup>A arm, this might indicate that an additional locus is found at 7H<sup>ch</sup>L, in agreement with durum wheat results. Nevertheless, since genetic mapping did not resolve *Psy1* position within chromosome 7H<sup>Ch</sup>, we cannot totally discard the occurrence of a translocation 7H<sup>A</sup>PL/7H<sup>Ch</sup>S during the development of the wheat/H. chilense chromosome addition line. Thus, macrocolinearity cannot be established for this gene at present and the YPC region on chromosome 7H<sup>ch</sup>L might possibly be related to *Psy1* variation. Indeed, *Psy1* showed a low association with YPC (*p* < 0.1).

Finally, there is a region on chromosome 2H<sup>ch</sup> related to YPC which extends over 80% of the chromosomal length. Four candidate genes, *Ggpps1*, *Zds*, *Hyd3* and *Ppo1*, are mapped within this interval. The most significant zone within this region is located in the distal part of chromosome 2H<sup>ch</sup>L (Figure 1). However, the complete KW-defined region must be considered as a target since the population size is probably hampering the determination of other QTLs in this chromosome. Indeed, in a preliminary map constructed using a H1×H2 F<sub>2</sub> population, which did not cover the distal part of chromosome 2H<sup>ch</sup>, a QTL for YPC was detected in the chromosome 2H<sup>ch</sup> in the vicinity of the marker *LaS-pHe2-1* [62] which is now mapped at 48 cM (Figure 1). Additionally, the location of four carotenoid-related genes in this area further supports the importance of this region for YPC.

The relevance of chromosome 2A for YPC and/or carotenoid content has been previously reported in wheat. Most works have focused on the role of polyphenol oxidases since these enzymes cause the darkening of flour, pasta and noodle products [63]. Duplicated polyphenol oxidase genes (*Ppo1* and *Ppo2*) have been identified and mapped clustered in barley 2H chromosome [64]. Although only *Ppo1* was mapped in *H. chilense*, a tightly linked *Ppo2* may be expected. In any case, the association of *Ppo1* and YPC in *H. chilense* seems to indicate the occurrence of darkening processes in this species. Similarly, the role of *Ppo-A1* and *Ppo-D1* has been extensively studied in wheat including mapping studies [65,66], the development of functional markers [67-69] and the cloning and analysis of these genes [70,71]. Recently, duplicated *Ppo* genes (*Ppo-A2*, *Ppo-B2* and *Ppo-D2*) have been identified [72] and mapped to chromosomes 2A, 2B and 2D [73]. Thus the results obtained in *H. chilense* are in agreement with previous findings in wheat.

Our results also suggest the potential importance of the centromeric region on chromosome 2H<sup>ch</sup> for YPC. QTL and association mapping studies have identified regions for YPC or carotenoid content in chromosome 2A [32,33,74]. Recently, a QTL on the centromeric region of chromosome 2A has been reported [33]. However, it could not be associated with YPC *per se* since the markers within the confidence interval were related to kernel weight variation. Thus, it was proposed that this QTL may be associated with yellow pigment concentration due to a lower carbohydrate content in smaller grains or it could be linked to a QTL for kernel weight [33]. Since there is no significant association between YPC and kernel weight in *H. chilense* [42], this region does not seem to be related to carotenoid concentration. Moreover, previous findings in durum wheat also support the existence of a QTL for YPC in this region [32,74]. Both studies showed the association between *Xgwm495* and YPC using a mapping population derived from the cross W9262-260D3 × Kofa [32] and a collection of 93 diverse accessions of durum wheat [74]. This marker is located in the arm 2AS (C-2ASS-078 bin) at 108.5 cM [75] (see Group 2 data file at http://wheat.pw.usda.gov/ggppages/SSRclub/GeneticPhysical/) between markers *Xgwm372* (C-2ASS-078 bin, at 90.8 cM) and *Xgwm249* (C-2AL-1.085 bin, at 111.9 cM). Both *Xgwm372* and *Xgwm249* are within the confidence interval for the QTL on chromosome 2A reported by [33]. Hence, all three works identify the same region around the centromere of chromosome 2A, and thus, this region seems to be effectively related to YPC and/or carotenoid content in durum wheat. The results presented in this work also support the importance of the centromeric region of the chromosome 2H<sup>ch</sup> in YPC. Furthermore, the *Zds* and *Ggpps1* genes were mapped within this region, and they should therefore be considered in durum wheat. The potential association of *Zds* with YPC in durum wheat has already been hypothesized after physical mapping on chromosomes 2A and 2B [76]. Later studies cloned *Zds* gene in common wheat [77] and identified a QTL for YPC related to *Zds-D1* variation in chromosome 2D in common wheat [78]. Similarly, the co-localization of *Zds* with a QTL for carotenoid content in chromosome 7 of maize [25] also supports the potential role of this gene in grasses. Similarly, the association between *Ggpps1* expression and the
carotenoid content in maize [29] and the close linkage between Zds and Ggpps1 in H. chilense make the latter an interesting candidate gene for durum wheat studies. Likewise, variations at Hyd3 locus are important for maize endosperm carotenoid content [28,79]. Given the association of this gene with YPC and its position in H. chilense map, Hyd3 should be also considered in further studies in durum wheat.

The most significant region of 2Hch, as identified by MQM analysis, is located in the distal part of the chromosome 2HchL where no candidate genes were located in this work. The occurrence of this association can be explained by several hypotheses, although they are all very speculative. The simplest one is that other carotenoid genes not considered in this study are located in the distal 2HchL. A second possibility would be that genes for carotenoid esterification are located in this region. A QTL for lutein esters was identified in chromosome 2B [35]. It is flanked by the marker KsuD22 which has been located in the chromosome 2BL by deletion mapping [75]. Esterification is a common mechanism for sequestering carotenoids in plants [80]. Indeed, carotenoids are accumulated in specialized lipoprotein-sequestering structures in the chromoplasts [81] and the apolar compounds of some chromoplasts, such as fibrils, are mainly composed of esterified xanthophylls [81]. H. chilense genome addition in tritordeums produces both high carotenoid content and an increase in lutein esterification [10], and, thus, the presence of genes for differential esterification ability in the 2HchL chromosome might be considered in the future.

The lack of association between the rest of the candidate genes (Dxr, Hdr, Ccd1, b-Lcy, Pds, Psy2 and Psy3) and YPC in H. chilense suggests at least two possibilities: (1) these genes are not related with YPC variation in the population; (2) they have minor effects which cannot be detected with the current population size. With the results presented in this work it is not possible to discern between these possibilities. While Psy2 and Psy3 are not associated with seed carotenoid content in other grasses (reviewed by [82]) and thus, they may be discarded for further analyses, the rest of the genes might be still useful for durum wheat studies. For instance, Ccd1 copy number is negatively correlated with carotenoid content in maize [83]. It is mapped in the distal part of 5HchS in concordance with QTLs for carotenoid content and YPC in durum wheat [33,84]. Thus, it may be an interesting candidate gene for durum wheat despite the lack of association with YPC in this work.

Conclusions

The equivalent location of the carotenoid-related genes in H. chilense and barley shows a high level of collinearity between both species, at least for the genes studied in this work. Considering the high synteny among Triticeae species along with the high YPC content of H. chilense and its derived amphiploids (tritordeums), the Hch genome constitutes a valuable tool for YPC and carotenoid content studies in the Triticeae. Indeed, the YPC regions detected in H. chilense showed good agreement with previous findings in wheat. Accordingly, the location of Zds and Ggpps1 within one of these regions around the 2Hch centromere is in concordance with QTL for YPC and carotenoid content in wheat, and suggests that both genes should be considered for durum wheat improvement. In addition, the identification and mapping of the candidate genes in H. chilense will enable further studies to investigate the genetic basis of the high YPC/carotenoid content in tritordeum which could also be extrapolated to durum wheat.

Additional file

Additional file 1: Table S1. Accession numbers of the sequences used for alignments in the design of primers for amplification of the orthologous genes in H. chilense.

Abbreviations

Dxr: 1-deoxy-D-xylulose 5-phosphate reductoisomerase; Hdr synonym ispH; 4-hydroxy-3-methylbut-2-enzyl diphosphate reductase; ACS: Automatic cofactor selection; Hyd3: Beta-carotene hydroxylase 3; Ccd1: Cleavage dioxygenase 1 gene; CAP: Cleaved Amplified Polymorphism; DART: Diversity Arrays Technology; Ggpps1: Geranyl geranyl pyrophosphate synthase; IM: Interval Mapping; KW: Kruskall-Wallis; b-Lcy: Lycopene beta-cyclase; e-Lcy: Lycopene epsilon cyclase; MEP: Methylerythritol phosphate; ML-mapping: Monte Carlo Maximum Likelihood Mapping algorithm; MQM: Multiple QTL Mapping; Pds: Phytoene dehydrogenase; Psy2: Phytoene synthase 2; Psy3: Phytoene synthase 3; Ppo1: Polyphenol oxidase 1 gene; QTL: Quantitative trait locus; RILs: Recombinant Inbred Lines; SNP: Single Nucleotide Polymorphisms; YPC: Yellow pigment content; Zds: Zeta-carotene desaturase.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

CR-S and SGA conceived and designed the study. CR-S performed the experiments. SGA performed the QTL analyses. CR-S and SGA analyzed the data and wrote the paper. Both authors have read and approved the final manuscript.

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