BMC Plant Biology

RESEARCH ARTICLE Open Access

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

Cristina Rodríguez-Suárez and Sergio G Atienza*

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 $2H^{ch}$, $3H^{ch}$ and $7H^{ch}$ 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 $2H^{ch}$ 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 H^{ch} 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, named tritordeums (×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 degradation [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

* Correspondence: sgatienza@ias.csic.es
Instituto de Agricultura Sostenible, IAS-CSIC, Apdo 4084, Córdoba E-14080, Spain

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endosperm carotenoid content using quantitative trait locus (QTL) and association studies. Indeed, several genes including *Psy1* [23-25]; *Zds* [25]; *e-Lcy* [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-Lcy*) 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 *Triticeae* due to the close relationship among these species. Indeed, *Triticeae* 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 *Triticeae*. 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 *Triticeae*. 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 F_7-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 $1H^{ch}$, $4H^{ch}$, $5H^{ch}$, $6H^{ch}$ or $7H^{ch}$ (named CS MA $1H^{ch}$-1 H^{ch}S, CS DA$4H^{ch}$, CS DA$5H^{ch}$, CS DA$6H^{ch}$ and CS DA$7H^{ch}$, respectively, where MA refers to monosomic addition and DA to disomic addition), and (CS)-*H. chilense* ditelosomic addition lines CS DA$1H^{ch}$S, CS DA$2H^{ch}$S, CS DA$5H^{ch}$L, CS DA$6H^{ch}$S, CS DA$7H^{ch}$α, CS DA$7H^{ch}$β.

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 Edialign 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 $MgCl_2$, 320 mM dNTPs (Promega, Madison, WI, USA), 0.6 mM of each primer and 50 ng of genomic DNA. PCR were carried out as follows: 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

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*, *NheI* 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 F_7-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*) and 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (*Hdr* [synonym *ispH*]) from the MEP pathway; geranyl geranyl pyrophosphate synthase (*Ggpps1*) for geranylgeranyl diphosphate synthesis; phytoene synthase 2 (*Psy2*), phytoene synthase 3 (*Psy3*), phytoene dehydrogenase (*Pds*), *zeta*-carotene desaturase (*Zds*), lycopene epsilon cyclase

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

Primer	Sequence 5′-3′	Primer	Sequence 5′-3′
GGPPS1-F1	GCCAGCGTCGACTCCTAC	PSY3-F	TCGACGAGCTGTACCTCTACTG
GGPPS1-R1	GGAACAGCAACCCAATTGAT	PSY3-R	CTCGATCTCGTCCAGGATCT
GGPPS1-spF	CGTATGCCTTTCTAAGAAGTG	HYD3-F3	CCTCCGTGTACTACCGCTTC
GGPPS1-spR	GGAGATACCTATGCAAATCAT	HYD3-R2	CGAACTTGTCCATGTGGTGT
DXR-F1	GAGCATGGGGAAGAAAATCA	ZDS-F2	TGTCCCAGGGATCAAAAGAC
DXR-R1	GGTGACCTCGGAGCAATAGA	ZDS-R2	AGCTTGGATCAGGGAACCTT
HDR-F2	GGCATTGCAAATCAAACAAC	LBC-F2	CAAGCTCAAGTCCACCATGA
HDR-R2	TTTCCTGGTCCAATCCTTTG	LBC-R2	TGTCTAGAAACCGCACGATG
LEC-F2	CTGGACAATATTTGCCTGGAA	LBC-spF	GCGACTCCCACCTCCCT
LEC-R2	GGAGGTGTCTGACGAGGTTC	LBC-spR	GATCGCGGACCCCTCG
PDS-F1	TACAGGTCGTGATTGCTGGT	CCD-F1	TTGATCCTACAAAGAAAGCTCGT
PDS-R1	GGGAAATCAAAACGGCTGTA	CCD-R1	CAGCTCATTCCCGAAGTTCT
PSY2-F	ATTGCTCCGGACTCAAAGG	CCD-spR	GAAGTTCTCCAGCTTGTCGG
PSY2-R	TTGTAGTCGTTCGCCTCGAT	PPO1-F1	AGCTTCGAGCAGCAGTGG
PSY2-spR	GAGTCAACAATGCTTGAATGA	PPO1-R1	GTGGTGCGCGAAGAAGAT

(*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 *Zds*, *Hyd3*, *Pds*, *Hdr*, *Psy3* and *e-Lcy* or to design allele-specific primers. Specific amplifications were obtained by using tetra-primer PCR (to amplify both H1 and H7 alleles) for *Ggpps1* (GGPPS1-F1/ GGPPS1-R1/ GGPPS1-spF/ GGPPS1-spR) and *b-Lcy* (LBC-F2/ LBC-R2/ LBC-spF/ LBC-spR). Alternatively, specific reverse primers CCD-spR and PSY2-spR were designed to use in combination with CCD-F1 and PSY2-F, respectively, to obtain PCR amplification exclusively in one of the parental alleles. Finally, length polymorphisms were detected for *Dxr* (14 bp insertion in H7 sequence compared to H1) and *Ppo1*. 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: $2H^{ch}$ (*Ggpps1*, *Zds*, *Hyd3* and *Ppo1*); $3H^{ch}$ (*Dxr* and *e-Lcy*); $4H^{ch}$ (*Hdr* and *Pds*); $5H^{ch}$ (*Ccd1*, *Psy2* and *Psy3*); $6H^{ch}$ (*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 $2H^{ch}$ at 142.38 cM and 161.56 cM respectively. The *e-Lcy* gene was located in the vicinity of the chromosome $3H^{ch}$ centromere while *Dxr* was mapped to the distal part of the $3H^{ch}S$ arm (Figure 1). *Hdr* and *Pds* genes were mapped to $4H^{ch}S$ and $4H^{ch}L$ arms respectively. *Ccd1* (distal) and *Psy2* (39.06 cM) were mapped to chromosome $5H^{ch}S$ 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 $6H^{ch}$ (111.2 cM) since the centromere is estimated at 84.0 cM [47]. Also, *Psy1* is located on chromosome arm $7H^{ch}\alpha$ 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

Table 2 Polymorphism between H1 and H7 lines used for mapping

Gene	Accession numbers		Polymorphism detection	
	H1	H7	Genetic mapping	Physical mapping
Dxr	JQ922078	JQ922079	Allele size	-
Hdr	JQ922096	JQ922097	MboII digestion	MboII digestion
Ggpps1	JQ922098	JQ922099	Allele specific primers	Allele specific primers
Psy2	JQ922084	JQ922085	Allele specific primers	Allele specific primers
Psy3	JQ922082	JQ922083	HhaI digestion	-
Pds	JQ922092	JQ922093	HhaI digestion	-
Zds	JQ922090	JQ922091	HinfI digestion	TasI digestion
e-Lcy	JQ922094	JQ922095	HhaI digestion	
b-Lcy	JQ922088	JQ922089	Allele specific primers	-
Hyd3	JQ922080	JQ922081	NheI digestion	-
Ccd1	JQ922086	JQ922087	Allele specific primers	Allele specific primers
Ppo1	JQ922103	JQ922105	Allele size	-
	JQ922104			

Polymorphism between H1 and H7 lines of *H. chilense* used for 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.

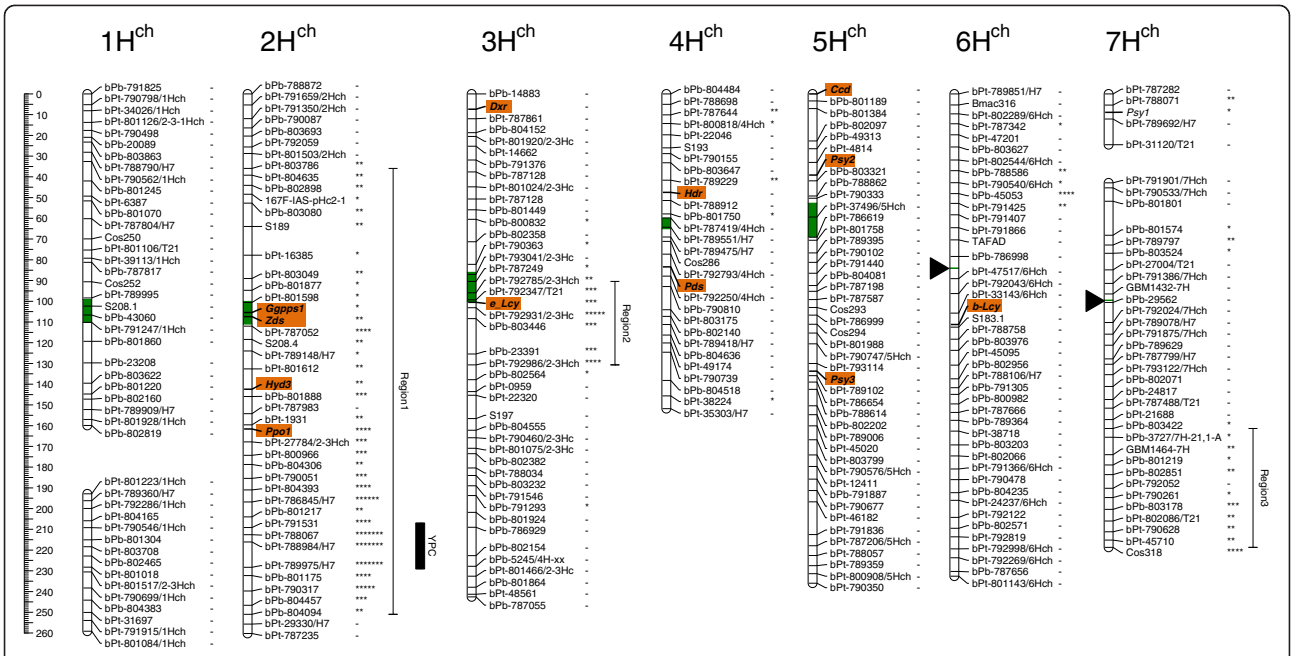

Figure 1 Marker-YPC association in the Kruskal-Wallis (KW) analysis. To determine marker-YPC associations, a framework map composed by markers spaced around 3–5 cM was considered from the map developed by [47]. The estimated centromere position is shown as a coloured segment within each chromosome. In chromosomes $6H^{ch}$ and $7H^{ch}$ centromeres were located in a single position since markers belonging to different chromosome arms mapped at the same position. For chromosomes $6H^{ch}$ and $7H^{ch}$, 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; *******0.0001. 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 $2H^{ch}L$) and its position estimated at the peak LOD with a 2-LOD support interval.

chromosomes $2H^{ch}L$ and $3H^{ch}$ (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 using the rice gene as query (Table 3). Orthologous genes in *Brachypodium* and *Sorghum* were identified by this means. The RAP locus identifier was retrieved using the ID Converter tool (http://rapdb.dna.affrc.go.jp/tools/converter). Rice, barley and sorghum orthologs were located in 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.

Determination of YPC regions in *H. chilense*

The YPC content was determined in the H1×H7 RIL mapping population. Kruskall-Wallis (KW) association test revealed three main chromosome regions related to YPC (Figure 1). The largest is located on chromosome $2H^{ch}$ where the majority of markers showed an association with YPC (Figure 1). Smaller regions were detected on chromosomes $3H^{ch}$ and $7H^{ch}$. Candidate genes *Ggpps1*, *Zds*, *Hyd3*, *Ppo1*, *e-Lcy* and *Psy1* showed a significant association with YPC (Figure 1). The MQM analysis resulted in a significant QTL at the distal part of chromosome $2H^{ch}$ between 207 and 229 cM ($p<0.05$) where no carotenoid-related genes were mapped in this work.

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

Table 3 Macrocolinearity between *H. chilense* and *H. vulgare* for the candidate genes selected

Gene	Identifier[1]	Rice LOC[2]	Rice RAP[3]	*Sorghum*[3]	*Brachypodium*[3]	*H. vulgare*[4]		*H. chilense*[5]	
						Chr	cM	Chr	cM
Ggpps1	POG_ORTHOMCL5142	LOC_Os07g39270	**Os07g0580900**	**Sb02g037510**	**Bradi1g23510**	2	59.21	$2H^{ch}C$	105.35
Zds	POG_ORTHOMCL7646	LOC_Os07g10490	Os07g0204900	Sb02g006100	Bradi1g54390	*nd*	*nd*	$2H^{ch}C$	107.36
Hyd3	POG_ORTHOMCL15690	LOC_Os04g48880	Os04g0578400	Sb06g026190	**Bradi5g19130**	2	90.1	$2H^{ch}L$	142.38
Ppo1	POG_ORTHOMCL2213	LOC_Os04g53300	Os04g0624650	**Sb06g025570/ Sb06g025580**	**Bradi5g22300**	2	117.9	$2H^{ch}L$	161.56
e-Lcy	POG_ORTHOMCL17555	LOC_Os01g39960	**Os01g0581300**	**Sb03g026020**	**Bradi2g41890**	3	55.57	$3H^{ch}C$-L	100.47
Dxr	POG_ORTHOMCL8746	LOC_Os01g01710	**Os01g0106900**	**Sb03g008650**	**Bradi2g00650**	3	0	$3H^{ch}S$	7.45
Pds	POG_ORTHOMCL8451	LOC_Os03g08570	**Os03g0184000**	Sb06g030030	**Bradi1g72400**	4	89.39	$4H^{ch}L$	83.39
Hdr	POG_ORTHOMCL5875	LOC_Os03g52170	Os03g0731850	**Sb01g009140**	**Bradi1g09710**	4	38.63	$4H^{ch}S$	47.49
Psy3	POG_ORTHOMCL18373	LOC_Os09g38320	**Os09g0555500**	**Sb02g032370**	**Bradi4g37520**	5	122.38	$5H^{ch}L$	133.75
Ccd1	POG_ORTHOMCL13689	LOC_Os12g44310	Os012g0640600	**Sb01g047540**	**Bradi4g00330**	5	0.0	$5H^{ch}S$	0.0
Psy2	POG_ORTHOMCL13713	LOC_Os12g43130	**Os12g0626400**	**Sb09g022310**	**Bradi4g01100**	5	26.28	$5H^{ch}S$	39.06
b-Lcy	POG_ORTHOMCL11455	LOC_Os02g09750	**Os02g0190600**	**Sb04g006120**	Bradi3g06600	6	54.6	$6H^{ch}L$	111.21
Psy1	POG_ORTHOMCL6706	LOC_Os06g51290	**Os06g0729000**	**Sb10g031020**	**Bradi1g29590**	7	166.56	$7H^{ch}\alpha$	8.93

([1]) *Poaceae* orthologous group corresponding to each candidate gene; ([2]) MSU rice database identifiers of rice genes used as query to identify the *Poaceae* orthologous group; ([3]) Rice, *Sorghum* and *Brachypodium* genes included in each *Poaceae* orthologous group. For nomenclature of rice genes (LOC vs RAP) see (http://rapdblegacy.dna.affrc.go.jp/gene/nomenclature.html); ([4]) Position of the candidate genes in barley according to the Barley Genome Zipper. ([5]) Position of the candidate genes in *H. chilense* map. Genes in bold are shown in the Barley Genome Zipper [56]; *nd*: not determined.

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 $7H^{ch}\alpha$ in *H. chilense* [31]. However, genetic mapping could not establish the relative position of *Psy1* in $7H^{ch}$ chromosome since it remained in a short linkage group not linked to $7H^{ch}$ chromosome. Thus, its putative location in $7H^{ch}\alpha$ 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 $7H^{ch}S$ 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 $7H^{ch}$.

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

The association study for YPC allowed the identification of three main regions in chromosomes $2H^{ch}$, $3H^{ch}$ and $7H^{ch}$ for YPC variation in *H. chilense* as revealed by a KW association test. Despite a single QTL at the distal part of chromosome $2H^{ch}L$ 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 $2H^{ch}$ 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 $3H^{ch}$ 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-Lcy* gene [25,26].

The distal part of chromosome $7H^{ch}L$ 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 $7H^{ch}\alpha$ arm, this might indicate that an additional locus is found at $7H^{ch}L$, in agreement with durum wheat results. Nevertheless, since genetic mapping did not resolve *Psy1* position within chromosome $7H^{ch}$, we cannot totally discard the occurrence of a translocation $7H^{ch}L/7H^{ch}S$ 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 $7H^{ch}L$ 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 $2H^{ch}$ related to YPC which extends over the 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 $2H^{ch}L$ (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×H7 F_2 population, which did not cover the distal part of chromosome $2H^{ch}$, a QTL for YPC was detected in the chromosome $2H^{ch}$ in the vicinity of the marker *IAS-pHc2-1* [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 $2H^{ch}$ 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-2AS5-078 bin) at 108.5 cM [75] (see Group 2 data file at http://wheat.pw.usda.gov/ggpages/SSRclub/GeneticPhysical/) between markers *Xgwm372* (C-2AS5-078 bin, at 90.8 cM) and *Xgwm249* (C-2AL-1-0.85 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 $2H^{ch}$ 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 $2H^{ch}$, as identified by MQM analysis, is located in the distal part of the chromosome $2H^{ch}L$ 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 $2H^{ch}L$. 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 $2H^{ch}L$ 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 $5H^{ch}S$ 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 H^{ch} 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 $2H^{ch}$ 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-enyl 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.

Acknowledgements

This research was supported by grants (to S.G. Atienza) AGL2011-24399 and P09-AGR-4817 from the Ministerio de Economía y Competitividad, Junta de Andalucía and FEDER. We are grateful to E. León and A. Pozo for their technical assistance and to C.M. Avila for the critical review of this manuscript. C. Rodríguez-Suárez acknowledges financial support from CSIC (JAE-Doc program). We acknowledge support of the publication fee by the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI).

Received: 4 September 2012 Accepted: 30 October 2012
Published: 2 November 2012

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doi:10.1186/1471-2229-12-200
Cite this article as: Rodríguez-Suárez and Atienza: *Hordeum chilense* genome, a useful tool to investigate the endosperm yellow pigment content in the *Triticeae*. *BMC Plant Biology* 2012 **12**:200.

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