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Quantitative trait loci governing carotenoid concentration and weight in seeds of chickpea (*Cicer arietinum* L.)

Abstract Chickpea is a staple protein source in many Asian and Middle Eastern countries. The seeds contain carotenoids such as beta-carotene, cryptoxanthin, lutein and zeaxanthin in amounts above the engineered beta-carotene-containing "golden rice" level. Thus, breeding for high carotenoid concentration in seeds is of nutritional, socio-economic, and economic importance. To study the genetics governing seed carotenoids in chickpea, we studied the relationship between seed weight and concentrations of beta-carotene and lutein by means of high-performance liquid chromatography in segregating progeny from a cross between an Israeli cultivar and wild *Cicer reticulatum* Ladiz. Seeds of the cross progeny varied with respect to their carotenoid concentration (heritability estimates ranged from 0.5 to 0.9), and a negative genetic correlation was found between mean seed weight and carotenoid concentration in the F_3. To determine the loci responsible for the genetic variation observed, the population was genotyped using 91 sequence tagged microsatellite site markers and two CytP450 markers to generate a genetic map consisting of nine linkage groups and a total length of 344.6 cM. Using quantitative data collected for beta-carotene and lutein concentration and seed weight of the seeds of the F_2 population, we were able to identify quantitative trait loci (QTLs) by interval mapping. At a LOD score of 2, four QTLs for beta-carotene concentration, a single QTL for lutein concentration and three QTLs for seed weight were detected. The results of this investigation may assist in improving the nutritional quality of chickpea.

Introduction

Chickpea (*Cicer arietinum* L.) is an Old World grain legume associated with the Neolithic origin of Near Eastern agriculture (Lev-Yadun et al. 2000). With an annual production of approximately 8 million tonnes, chickpea ranks third among the world's pulse crops (FAO 2003). The Indian sub-continent accounts for about 80% of the global production, while eastern Africa, the Mediterranean and Near East countries, Australia, southern Europe and North and South America contribute the rest. Being a staple diet component, chickpea supplies starch and protein to the predominantly vegetarian population in India and is considered to be a health food in Western countries. Thus, the nutritional value of chickpea is highly important in maintaining human health in developing countries and may serve as a marketing tool in the West. As was recently shown with the development of "golden rice", increasing the carotenoid concentration of staple foods provides an appreciable opportunity to improve food quality and subsequently the health of low-income population sectors in the third world (Ye et al. 2000). However, instead of applying expensive approaches to increase the concentration of carotenoids by genetic engineering, it is more economical and more easily accepted by critical consumers to explore and exploit the

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available genetic variation and its underlying genetic basis in the primary and secondary gene pools of current staple crops.

Carotenoids comprise a group of more than 600 naturally occurring lipophilic pigments, at least 50 of which occur in plant foods. Two groups of carotenoids are found in human blood: the hydrocarbon carotenoids—such as alpha- and beta-carotene, and lycopene—and the xanthophylls—such as lutein, zeaxanthin and beta-cryptoxanthin. The discrepancy between the number of carotenoids in the diet and those present in plasma clearly speaks for selective uptake. Of all the carotenoids, beta-carotene is the most widely distributed in plants and the one most efficiently converted to vitamin A. The A family of fat-soluble vitamins (including retinol, retinal and retinoic acid) plays an important role in vision, bone growth, reproduction, cell division and cell differentiation in mammals. For example, at least 3 million children in Third World countries develop xerophthalmia (damage to the cornea of the eye), and 250,000–500,000 become blind each year because of vitamin A deficiency (Reifen 2002).

Over the past few decades, several new functions for carotenoids have been identified. Beta-carotene, which protects against coronary artery disease (Gey et al. 1993), is the best-studied of the carotenoids. However, increasing interest is being given the other carotenoids, such as lycopene, as a possible chemoprotectant against prostate cancer (Giovannucci et al. 1995), and lutein and zeaxanthin, being the only carotenoids present in the macula region of the retina where they are effective against senile macular degeneration. Carotenoids serve here as antioxidants due to their ability to inactivate singlet oxygen and to quench carboxyl radicals in vitro (Meydani et al. 1994); they also can function as blue light filters (Khachik et al. 1997). Thus, individuals with low carotenoid intake and/or low body carotenoid levels suffer from increased risk of degenerative diseases and certain cancers (Ziegler 1989). Carotenoids also benefit the immune system as they reduce UV-induced immunosuppression and increase natural killer cell activity (Santos et al. 1998). Moreover, beta-carotene, cantaxanthin and cryptoxanthin efficiently induce gap-junction communication in cell-to-cell interactions, which is important for morphogenesis and cell differentiation (Stahl and Sies 1998).

Therefore, from both a purely nutritional point of view and socio-economic considerations (staple role of chickpea in highly populated countries) any information that may assist in improving the nutritional value of chickpea is important (e.g. Abbo et al. 2000). In this context, an understanding of the genetic basis of carotenoid concentration is highly relevant.

The aims of the investigation reported here were to identify genotypes suitable for breeding and to genetically map quantitative trait loci (QTLs) controlling carotenoid concentration in seeds and to detect sequence tagged microsatellite site (STMS) markers useful as tags for these traits for breeding. Further, the map locations of the putative loci should be related to the detailed interspecific genetic map of chickpea (Winter et al. 2000) to make these results available to the chickpea breeding community and to evaluate their potential for future marker-assisted selection and map-based cloning of agronomically important genes. We determined seed weight and carotenoid concentration in seeds of a segregating population from a cross between wild and domesticated chickpea genotypes and subsequently mapped QTLs responsible for carotenoid concentration in seeds using anchor-STMS markers.

Materials and methods

Plant material

To analyse the inheritance of carotenoid concentration in chickpea seeds, we crossed a cultivated chickpea (*Cicer arietinum* L.) variety (cv. *Hadas*, kabuli type) with a wild *C. reticulatum* accession (Cr205). *Hadas* is a high-yielding, modern Israeli cultivar, while *C. reticulatum* Cr205 originates from Mardin province, southeastern Turkey. A wide cross was chosen in order to exploit the greater genetic variability between the species for improved genetic mapping. The hybrid nature of the F_1 plants was confirmed by the presence of dominant alleles controlling flower colour and stem pigmentation contributed by the male parent Cr205. Since the analysis of carotenoid concentration requires destructive procedures, no attempt was made to evaluate the carotenoid concentration of the F_2 seeds.

Two sets of progeny derived from the above cross were studied. One set of the *Hadas* × Cr205 F_2 progeny were grown without any DNA or seed carotenoid analyses. To allow a replicated experiment at the F_3 generation, F_3 seeds derived from 46 individual F_2 plants (of the first set) were planted in January 2000, in Rehovot, Israel. The nursery was set up inside the screen-house along trickle-drip irrigation lines (40 cm between the rows and 2 m between the irrigation lines), at a density of 8 plants m^{-1} within the row, on Rhodoxeralf soil. The plants were irrigated until the pods of the latest maturing genotypes had fully developed. Additional husbandry details were as described by Or et al. (1999). The experiment was designed as follows: groups of four plants from each genotype (F_3 family or parent) were randomly arranged along the irrigation lines. A total of 46 F_3 families, each derived from individual F_2 plants, were planted with three replicates (four plants per replicate) of each family. The only exceptions were a single family with two replicates only (due to limited F_3 seeds) and a single family with four replicates. Six replicates (four plants each) of Cr205 and 12 replicates of cv. *Hadas* were planted. At full maturity, F_3 plants were harvested and thrashed individually, and their F_4 seeds counted and weighed. Seeds from up to eight individuals of each of the F_3 families were taken for chemical (lutein, zeaxanthin, beta-cryptoxanthin,

lycopene, alpha-carotene, beta-carotene) analyses (a mean number of 5.6 individuals per F_3 family).

The second set of 120 F_2 individuals (independent of the first set) was used for the genetic mapping of carotenoid QTLs. The F_2 seeds were obtained from the selfing of an F_1 hybrid from the same cross combination (cv. *Hadas* × Cr205) that was grown during the 2000 season. These F_2 progeny were grown in pots and their vegetative tissue sampled for DNA extraction. The F_2 plants were grown in a 50-mesh screenhouse in Rehovot, Israel (during the 2001 growing season), in 5-l pots and irrigated until the last individual fully matured. The potting mixture contained tuff, peat and vermiculite in a 1:1:1 ratio. Each pot was supplemented with 5 g of NPK (and microelements) slow-release fertilizer. All pots were connected to a computer-controlled trickle irrigation system. No signs of water or mineral deficiency were observed on any of the plants. Samples of ripe F_3 seeds from each individual F_2 plant were later analysed for their carotenoid concentrations (the mapping population). The DNA polymorphism and seed carotenoid concentrations (lutein, lycopene, beta-carotene) were later subjected to QTL analysis (details below).

High-performance liquid chromatography (HPLC) analysis of carotenoid concentration

Chemicals (technical grade or higher) were purchased from Merck (Darmstadt, Germany), or JT Baker (Deventer, The Netherlands, and Haifa, Israel). Chromatography solvents (HPLC-grade) were also obtained from JT Baker. Carotenoid standards were obtained from Sigma (St. Louis, Mo.). Samples were injected by using the 100 μl-loop on a JASCO Autosampler (model AS-950-10; JASCO, Tokyo, Japan) onto a C18, RP (Vydac 201TP45; Bucher Biotec, Basel, Switzerland) column. Samples were eluted isocratically in the HPLC mobile phase at a flow rate of 1.2 ml min^{-1} with a model LC-1150 pump (GBC, Scientific Equipment, Victoria, Australia), a multiwave programmable detector (model MD 910, JASCO) and a Borwin PDA version 1.50 system controller (JASCO). Carotenoids were identified and quantified at 450 nm against known standards. Statistical analyses of carotenoid data were performed either with EXCEL (ver. 98, Microsoft, Redmond, Wash.) or with JMP software (Sall and Lehman 1996).

DNA isolation

For genetic mapping, DNA was isolated from 2.5 g fresh leaves from parental lines and progeny of the cross using a modified CTAB method (Weising et al. 1995). After the re-suspension of DNA pellets in 5 ml of sterile double distilled water, residual polysaccharides were precipitated according to Michaels et al. (1994). Since the amount of DNA from F_2 individuals was low, 12–15 F_3 plants derived from a single F_2 plant were grown in the greenhouse in Frankfurt, Germany. Exactly 0.2 g mature leaves of each of the 12–15 individual plants per F_3 family were bulked and their DNA extracted. To increase the number of STMS anchor loci common to the *Hadas* × *C. reticulatum* map and to the map of Winter et al. (1999, 2000), we also isolated DNA from 130 F_9 recombinant inbred lines (RILs) from a cross ICC4958 × *C. reticulatum* PI 489777 employed for the generation of a high-density interspecific map (Winter et al. 1999, 2000). The DNA samples of the F_9 RILs were used to locate new STMS markers not present in the map for this population.

DNA markers and amplification conditions

Initially, 271 STMS primer pairs (Hüttel et al. 1999; Winter et al. 1999) were tested for their potential to detect polymorphisms between the parental lines of the *Hadas* × *C. reticulatum* cross. Informative markers were used to amplify by PCR the DNA either from bulked F_3 leaves or from F_2 individuals. Amplifications were carried out as described by Hüttel et al. (1999 and Winter et al. (1999). Amplification products were separated on 2.5% agarose gels and stained with ethidium bromide. In the case of very small size differences between alleles, products were electrophoresed on 30 × 40-cm 8% nondenaturing, vertical polyacrylamide gels and again stained with ethidium bromide. STMS markers detecting polymorphisms between the parental lines were used to screen the entire segregating population of 120 F_2 progeny. To increase the comparability of the map with that of Winter et al. (1999), markers displaying polymorphisms between the parental lines of the population used by Winter et al. (1999, 2000) and which had not been mapped before were additionally mapped in the ICC4958 × PI 489777 RIL progeny.

In a second approach, markers were developed using 22 sets of primers derived from conserved domains of different subfamilies of cytochrome p450 (CytP450) genes either in combinations of subfamily-specific primers or in combinations with anchored or unanchored primers containing microsatellite sequences (primer sequences available on request). The amplifications were performed on 15–20 ng DNA with 10 pmol of each primer, 200 μM dNTPs, 0.4 U *Taq* polymerase (Genecraft, Germany) in a buffer provided by the supplier containing 1.5 m M MgCl$_2$. Amplification was initiated with an initial denaturation step of 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 1 min, elongation at 72°C for 1 min, with a final extension step at 72°C for 4 min. For radioactive detection, one of the two primer oligonucleotides of each primer combination was labelled with 0.0185 MBq [^{32}P]-dATP by incubation with polynucleotide kinase (Fermentas, St. Leon-Rot, Germany) in buffer provided by the supplier. A touch-down PCR protocol was applied, starting with the initial denaturation step at 94°C for 2 min, followed by 14 touch-down

cycles consisting of denaturation at 94°C for 40 s, annealing temperatures decreasing by 0.7°C per cycle from 65°C to 55°C for 1 min and elongation at 72°C for 1 min. For an additional 25 cycles the annealing temperature was kept constant at 55°C. The final elongation step at 72°C was for 4 min. Amplification products were separated on 6% denaturing polyacrylamide gels. Gels were exposed to X-ray film overnight. Primer combinations amplifying polymorphic amplicons from the parental lines were employed to amplify the DNA of six individuals of the segregating population to test whether the marker was dominant or co-dominant. Co-dominant markers were used to amplify the DNA of the whole population.

Goodness-of-fit tests, linkage analysis
and mapping of QTLs

Goodness-of-fit to the 1:2:1 segregation ratio expected for the segregation of genes in F_2 generations was carried out by chi-square tests using the programme STAT-ISTICA (StatSoft 1999). Linkage analysis was performed with MAPMAKER ver. 3.0b (Lander et al. 1987). Markers were assigned to linkage groups (LGs) at a minimum LOD score of 4.0. Marker order and distances between markers were calculated using the COMPARE command and MAPMAKER's KOSAMBI algorithm (Kosambi 1944). Marker order was corrected with MAPMAKER's TRY routine. The resulting order of markers was fed into the programme MAPQTL ver. 4.0 (Van Ooijen et al. 2002) for QTL analysis. QTLs were mapped using the INTERVAL MAPPING routine (Lander and Botstein 1989; Van Ooijen 1992) based on a maximum likelihood approach to the segregation of a mixture of probability distributions (McLachland and Basford 1988).

Results

Genetics of carotenoid concentration

Mean seed weight values and mean carotenoid concentration of the two parental genotypes are given in Table 1. Mean seed weight of the cultivated genotype was higher than that of the wild accession Cr205, whereas the wild accession's seeds contained higher concentrations of the measured carotenoids.

Of each F_3 family, four to six individuals were randomly selected for analysis of their seeds' carotenoid concentration. Data were subjected to a one-way anal-

ysis of variance (ANOVA) with the F_3 family as the class variable. F values of the F_3 family factor for seed weight, mean lutein concentration, mean zeaxanthin concentration, mean beta-cryptoxanthin concentration and mean alpha- and beta-carotene concentration were highly significant with $P(F) < 0.0001$, as was expected from the differences between the parental genotypes (Table 1).

Narrow-sense heritability (h^2) values of the analysed traits were calculated using the between- and within-F_3 family variance components as recommended by Cahaner and Hillel (1980). In the absence of a significant F_3 family variance component (more than 93% of the variation was within the F_3 families) for lycopene, no heritability estimate was calculated for this trait. The h^2 values given in Table 2 indicate that all of the other traits analysed have relatively high heritability, as was expected from the significant variance component between the F_3 families.

The correlation between the analysed traits based on F_3 family means are shown (above the diagonal) in Table 3. These values provide a good approximation for the genetic correlation between the traits since they are based on family means. The calculated genetic correlation (r_G) based on the between-F_3 family covariance [below the diagonal, Table 3; after Cahaner and Hillel (1980)], are in very good accordance with the estimates based on family means. The relationship between mean seed weight, and beta-carotene, zeaxanthin and lutein concentration (also based on F_3 family means) is presented in Fig. 1.

Genetic mapping of QTL for carotenoid
concentration

Of the 271 STMS primer pairs and 22 CytP450-derived primer combinations initially tested, 91 markers detected polymorphisms between the parental lines and were used to screen the F_2 progeny, which in most cases was represented by bulked F_3 DNAs. To test the reliability of the grouping method for re-constructing the allelic state of the F_2, DNA of individuals for which both the F_2 and bulked F_3s were available was amplified with the same STMS primer pairs. In all cases, loci heterozygous in the F_2 gave rise to two bands in the DNA from the bulked F_3, as was expected. The typical segregation pattern of a STMS locus amplified from DNA derived from the bulked F_3s is shown in Fig. 2.

A total of 22 combinations of primers specific to sequences conserved in cytochrome p450 subfamilies,

Table 1 Mean seed weight and mean carotenoid concentration (ug g^{-1} seed dry weight) of the two chickpea parental lines ($\pm$ standard deviation) during the 2000 experiment

Genotype	Mean grain weight (g)	Lutein	Zeaxanthin	beta-Cryptoxanthin	Lycopene	alpha-Carotene	beta-Carotene
Hadas	0.56±0.008	2.75±0.06	1.6±0.1	0.014±0.001	0.003±0.0003	0.025±0.001	0.096±0.03
Cr205	0.18±0.026	6.22±0.29	2.27±0.16	0.038±0.013	0.003±0.0009	0.075±0.005	0.48±0.05

Table 2 Heritability values (± standard error) based on the between- and within-F_3 variance components of the analysed traits in the cross *Hadas* × Cr205

Trait	Heritability
Seed weight	0.71 ± 0.18
Lutein concentration	0.91 ± 0.21
Zeaxanthin concentration	0.68 ± 0.18
beta-Cryptoxanthin	0.53 ± 0.15
alpha-Carotene concentration	0.82 ± 0.2
beta-Carotene concentration	0.86 ± 0.2

either in combination with microsatellite and anchored microsatellite primers or in combinations with each other, amplified approximately 220 clearly distinguishable amplicons from the parental lines. Of these, 46 were polymorphic between the parents, but only five were codominant and could be employed to amplify the segregating population. Due to ambiguities in scoring the heterozygotes in the bulked F_3, only two markers remained (CYPE3 and CYPE5) that could be reliably scored in the population (data not shown).

The genetic map of the *Hadas* × *C. reticulatum* cross and its comparison to previous genetic maps of chickpea

From the 93 polymorphic markers, 72 could be arranged into nine LGs comprising 344.6 cM (Fig. 3). Twenty-one markers were unlinked. Co-linearity of the present map with the previously published interspecific STMS marker map of the chickpea genome (Winter et al. 1999) was established using STMS markers common to both maps as anchors. To increase the number of markers common to both maps, we mapped additional STMS markers in the RIL population used by Winter et al. (1999, 2000) and updated the map (Fig. 3). One LG (LG 5) of the previous map was split into two groups (LGs 5B-1and 5B-2) in the present map, while the marker order in the other eight LGs is almost co-linear, despite a few small differences in linear order and the distances between them (Fig. 3). The segregation of ten markers deviated significantly ($P > 0.05$) from the expected 1:2:1 ratio. Eight of these were located in a single

LG (on LG 4B: markers GA2, GA24, TA186, TR20s, TS54, TS104 and STMS11, Fig. 3).

Location of QTLs for seed weight and carotenoid concentration on the genetic map of chickpea

Only QTLs with LOD scores of greater than 2 were considered in the present work. The location of QTLs for the different traits on the genetic map, their LOD scores and the most closely linked markers flanking the QTLs are summarized in Table 4. For beta-carotene concentration, four QTLs (Fig. 3), including the most significant one (LOD 3.8) associated with the unlinked marker TS19 and a less significant QTL linked to marker TR26 (LOD 2.1), were detected. Although both markers are part of LG 3 in the map of Winter et al. (2000), they were not associated with LG 3 in the present study. Interestingly, QTL2 for beta-carotene concentration resides between these two markers on the same LG. For lutein concentration, only a single QTL on LG 8 was found (Fig. 3). The most significant QTL for seed weight (Fig. 3) was located on LG 4B flanked by markers GA24 and STMS11 on one side and GA2 on the other. Two other, less significant seed weight QTLs were detected on LG 1B (QTL2) and 2B (QTL3). Note that QTL2 for seed weight and QTL3 for beta-carotene concentration are both linked to marker GA11 on LG 1B, although they do not overlap (Fig. 3).

Discussion

Prospects for improving the supply of nutritional carotenoid with chickpea seeds

In general, the major carotenoids in chickpea seeds are lutein, zeaxanthin and, to a lesser extent, beta-carotene, with beta-cryptoxanthin, lycopene and alpha-carotene present in much smaller quantities. Mean concentrations of these carotenoids are higher in the wild accession than in its cultivated counterpart, cv. *Hadas*, except for lycopene, which is present in equally small amounts in both genotypes. It is worth noting that, despite the variation observed, carotenoid concentration in dry

Table 3 Correlation coefficients[a] and calculated genetic correlations[a] between the analysed traits in the *Hadas* × Cr205 population

	Mean seed weight	Lutein	Zeaxanthin	beta-Cryptoxanthin	Lycopene	alpha-Carotene	beta-Carotene
Mean seed weight		**-0.33**	**-0.36**	−0.15	−0.20	0.15	**-0.59**
Lutein	−0.19			0.27	−0.09	0.27	**0.58**
Zeaxanthin	−0.36	**0.66**		**0.39**	0.04	−0.03	**0.47**
beta-Cryptoxanthin			0.39		0.02	−0.14	**0.39**
Lycopene						0.10	0.14
alpha-Carotene							−0.12
beta-Carotene	−0.61	0.58	0.2	0.43			

[a]Values are based on the mean values of the F_3 families (above the diagonal) and the calculated genetic correlations (r_G; below the diagonal). Coefficients that differ from zero at $P < 0.05$ are depicted in bold
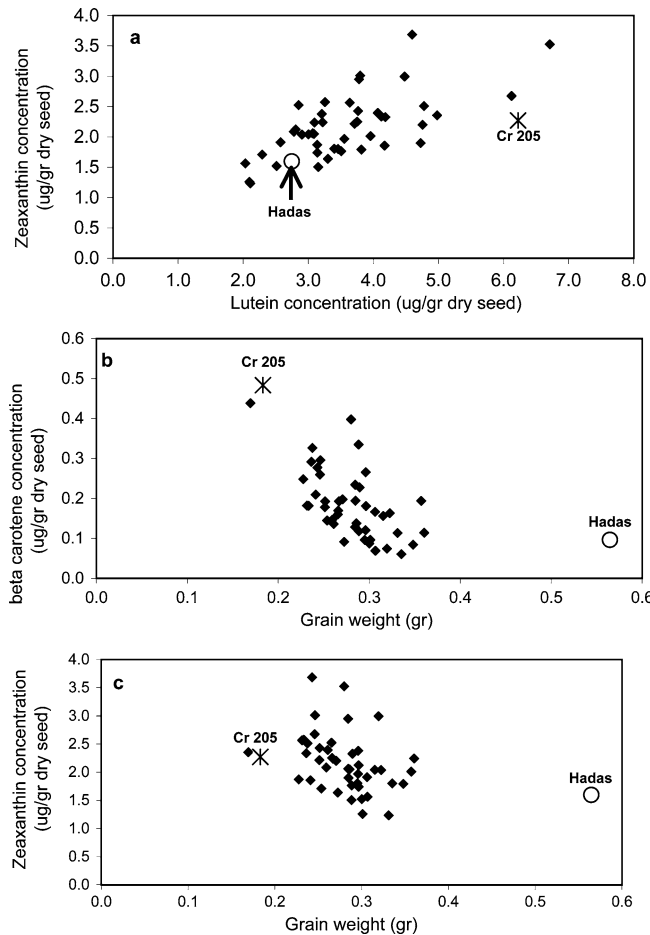
Fig. 1 The relationship between mean carotenoid concentration and seed weight in an F_3 population from the cross cv. *Hadas* × *Cicer reticulatum*. **a–c** The relationship between: **a** mean zeaxanthin concentration and mean lutein concentration, **b** mean beta-carotene concentration and mean seed weight, **c** mean zeaxanthin concentration and mean seed weight
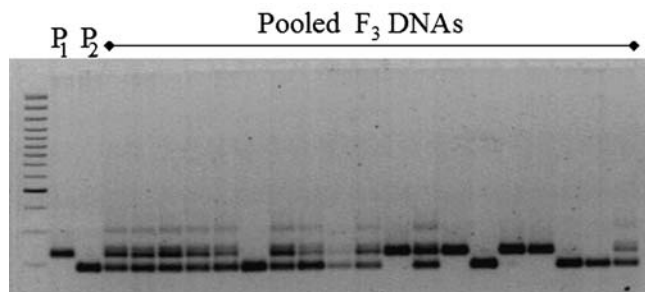
Fig. 2 Representative image of STMS marker bands (STMS 110) segregating in an F_2 population from cross *Hadas* × Cr205. Photograph of an e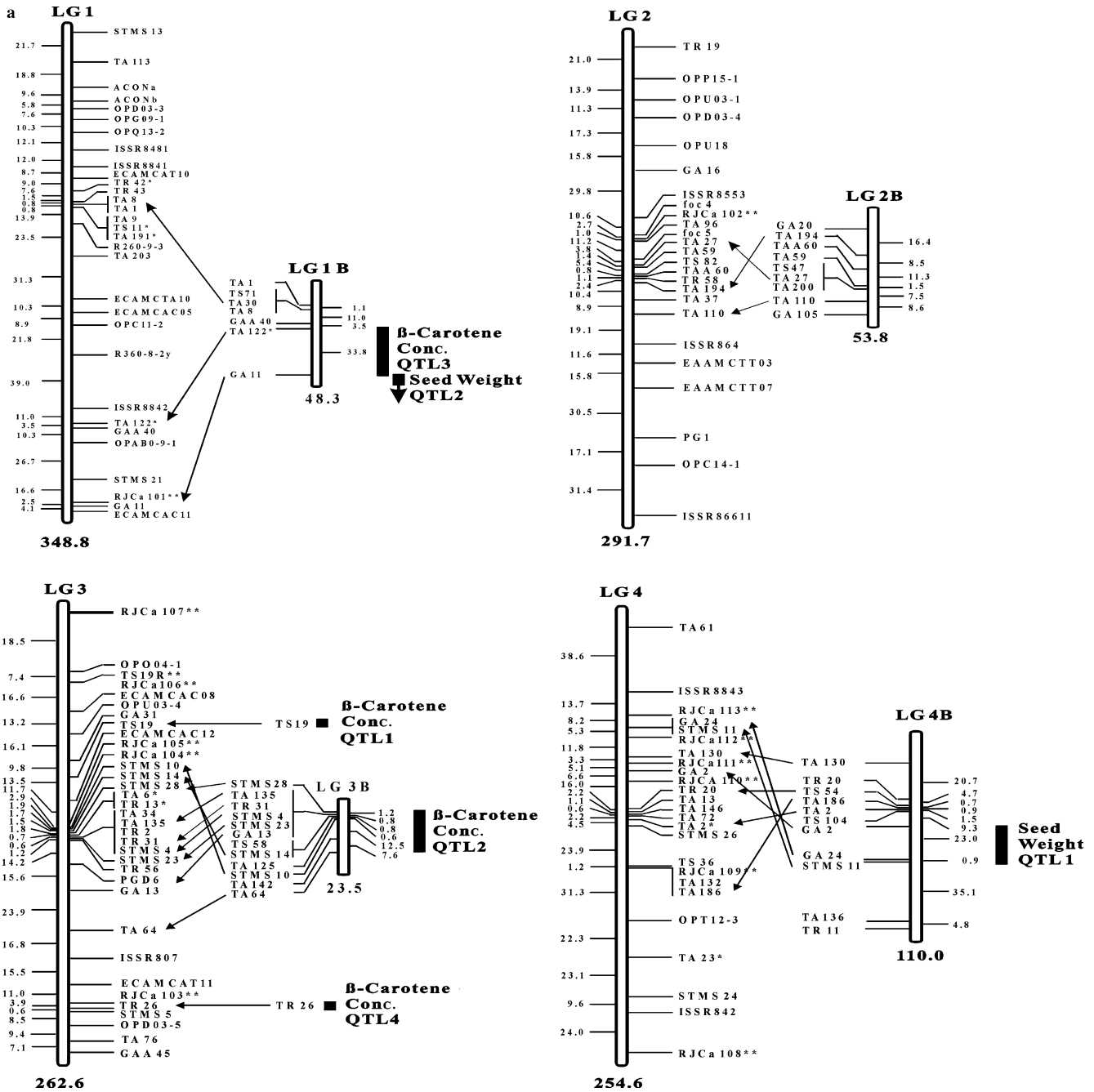thidium bromide-stained 2.7% agarose gel. Lanes P_1 (cv. Hadas) and P_2 (Cr205) contain the amplification products from the parental lines (230 bp and 190 bp, respectively); the other lanes contain amplification products of bulked F_3DNAs derived from individual F_2 plants (see text for more details). Note that heterozygous and homozygous pools can be clearly distinguished. The molecular weight marker used is a 100-bp ladder (MBI-Fermentas, Vilnius, Lithuania)

▶

Fig. 3 Comparative interspecific DNA marker maps from a RIL population from the cross ICC4958 × *C. reticulatum* PI489777 represented by linkage groups (*LGs*) without extension and from the cross *Hadas* × Cr205 represented by LGs with extension B (as, for example, LG 1B). The relative positions of markers segregating in both populations are indicated by *arrows*. The *number at the bottom* of each LG as well as at the *right* of the LGs (on the *left* of LGs from the F_2 population) determined in the RILs give the genetic distance between adjacent markers or the added genetic distances for the whole linkage group, respectively, in centiMorgans (cM). From the 425 markers available to date, only STMS markers and framework markers covering the entire LGs are shown for clarity. Note that in the *Hadas* × Cr205 map several STMS markers are present that could not be mapped in the RIL population due to the lack of polymorphism in this population. The positions of significant QTLs for beta-carotene and lutein concentration as well as those for seed weight are indicated by *bars* labelled with the respective name of the QTL (see Table 4)

chickpea seeds was always by far higher than that in dry "golden rice" endosperm (1.6 µg g⁻¹; Ye et al. 2000). In countries where chickpea is a dominant staple food, and fruits and other vegetables are scarce or expensive, chickpea would therefore be a much superior source of dietary carotenoids than "golden rice" due to the higher concentration of these pigments in the former. Moreover, further selection and breeding could increase the level of dietary carotenoid in an average serving of chickpea to close to the recommended daily allowance level for pro-vitamin A carotenoids. Since relatively high concentrations of lutein, zeaxanthin and beta-carotene were documented in chickpea seeds in this study, it might be interesting to screen more (wild and cultivated) chickpea accessions for their carotenoid concentrations. Of special importance would be *C. reticulatum* and *C. echinospermum*, both of which are cross compatible with cultivated chickpea. In such a way, one could identify novel sources of high-carotenoid alleles and explore their breeding potential to broaden the present gene pool.

Genetics of carotenoid concentration in chickpea seeds

The significant variation between plants of the F_3 family confirms that genes governing seed carotenoid concentrations segregate in the studied population. High h^2 values, as observed for lutein (0.91) and beta-carotene (0.86), suggest that only a few genes are involved in controlling the concentrations of these two carotenoids. The genetic correlations between the various carotenoid concentrations are positive (Table 3). The relationship between mean lutein concentration and mean zeaxanthin concentration shows transgressive segregation for both traits. As depicted in Fig. 1c, some families show lower values than the low-concentration parent *Hadas* for both parameters, while other families show higher values for both parameters relative to the high-producing parent Cr205. This result implies that it should be possible to improve both lutein and zeaxanthin concentration values beyond those of the parental genotypes.

a **LG 1**

21.7	STMS 13
	TA 113
18.8	
9.6	ACONa
5.8	ACONb
7.6	OPD03-3
	OPG09-1
10.3	OPQ13-2
12.1	ISSR8481
12.0	ISSR8841
8.7	ECAMCAT10
9.0	TR42*
7.6	TR43
1.5	TA8
0.8	TA1
13.9	TA9
	TS11*
	TA191*
23.5	R260-9-3
	TA203
31.3	
	ECAMCTA10
10.3	ECAMCAC05
8.9	OPC11-2
21.8	
	R360-8-2y
39.0	
	ISSR8842
11.0	TA122*
3.5	GAA40
10.3	OPABO-9-1
26.7	
	STMS21
16.6	RJCa101**
2.5	GA11
4.1	ECAMCAC11

348.8

LG 1B

TA 1 / TS71 / TA30 / TA8 / GAA 40 / TA 122* — 1.1 / 11.0 / 3.5 / 33.8
GA 11
48.3

ß-Carotene Conc. QTL3
Seed Weight QTL2

LG 2

21.0	TR 19
13.9	OPP15-1
11.3	OPU03-1
17.3	OPD03-4
15.8	OPU18
	GA 16
29.8	ISSR8553
10.6	foc 4
2.7	RJCa 102**
1.0	TA 96
11.2	foc 5
	TA 27
	TA 59
	TS 82
	TAA 60
	TR 58
	TA 194
10.4	TA 37
8.9	TA 110
19.1	ISSR864
11.6	EAAMCTT03
15.8	EAAMCTT07
30.5	
	PG 1
17.1	OPC14-1
31.4	ISSR86611

291.7

LG 2B

GA20 / TA 194 / TAA60 / TA59 / TS47 / TA 27 / TA 200 / TA 110 / GA 105
16.4 / 8.5 / 11.3 / 1.5 / 7.5 / 8.6
53.8

LG 3

	RJCa 107**
18.5	
7.4	OPO04-1
	TS19R**
16.6	RJCa106**
	ECAMCAC08
	OPU03-4
13.2	GA31
	TS19
16.1	ECAMCAC12
	RJCa105**
9.8	RJCa104**
13.5	STMS 10
11.7	STMS 14
2.9	STMS 28
1.9	TA 6*
1.7	TR 13*
1.3	TA 34
1.5	TA 135
1.8	TA 2
0.7	TR 31
0.6	STMS 4
14.2	STMS 23
15.6	TR 56
	PGD6
	GA 13
23.9	
	TA 64
16.8	
	ISSR807
15.5	
	ECAMCAT11
11.0	RJCa103**
3.9	TR 26
0.6	STMS 5
8.5	OPD03-5
9.4	TA 76
7.1	GAA 45

262.6

LG 3B

STMS28 / TA 135 / TR 31 / STMS 4 / STMS 23 / GA 13 / TS 58 / STMS 14 / TA 125 / STMS 10 / TA 142 / TA 64
1.2 / 0.8 / 0.8 / 0.6 / 12.5 / 7.6
23.5

ß-Carotene Conc. QTL1 (TS19)
ß-Carotene Conc. QTL2
ß-Carotene Conc. QTL4 (TR 26)

LG 4

38.6	TA 61
	ISSR8843
13.7	RJCa 113**
8.2	GA 24
5.3	STMS 11
	RJCa112**
11.8	TA 130
3.3	RJCa111**
5.1	GA 2
6.6	RJCa 110**
16.0	TR 20
2.2	TA 146
1.1	TA 72
0.6	TA 2*
2.2	STMS 26
4.5	
23.9	TS 36
1.2	RJCa 109*
	TA 132
31.3	TA 186
22.3	OPT12-3
23.1	TA 23*
9.6	STMS 24
	ISSR842
24.0	RJCa 108**

254.6

LG 4B

TA 130 / TR 20 / TS 54 / TA 186 / TA 2 / TS 104 / GA 2
20.7 / 4.7 / 0.7 / 0.9 / 1.5 / 9.3 / 23.0
GA 24 / STMS 11 — 0.9
TA 136 / TR 11 — 35.1 / 4.8
110.0

Seed Weight QTL1

Correlation between seed weight and carotenoid concentration of chickpea seeds

Any commercial breeding programme has to take into account that large seed size is of eminent importance for the acceptance of chickpeas by consumers. Thus, the significant negative correlation between mean seed weight and relatively high levels of carotenoids, namely lutein, zeaxanthin, and beta-carotene (Table 3), is a potential obstacle for Kabuli chickpea breeders, because attempts to improve carotenoid concentration may eventually force a certain reduction in mean seed weight. To overcome this problem, advanced breeding techniques such as marker-assisted breeding could help select for promoting alleles to improve both characters. Lycopene and alpha-carotene concentrations were not correlated with mean seed weight (Table 3), probably due to their relatively narrow range. The scatterplot of the F_3 family means of seed weight and beta-carotene (Fig. 1) shows that most of the families had values that were lower than the means of the parental values for both parameters (0.289 μg g^{-1} DW and 0.37 g for beta-carotene and seed weight, respectively). In addition, hardly any family had a mean beta-carotene concentration similar to that of the Cr205 parent, while many families resembled the *Hadas* parent. This may be
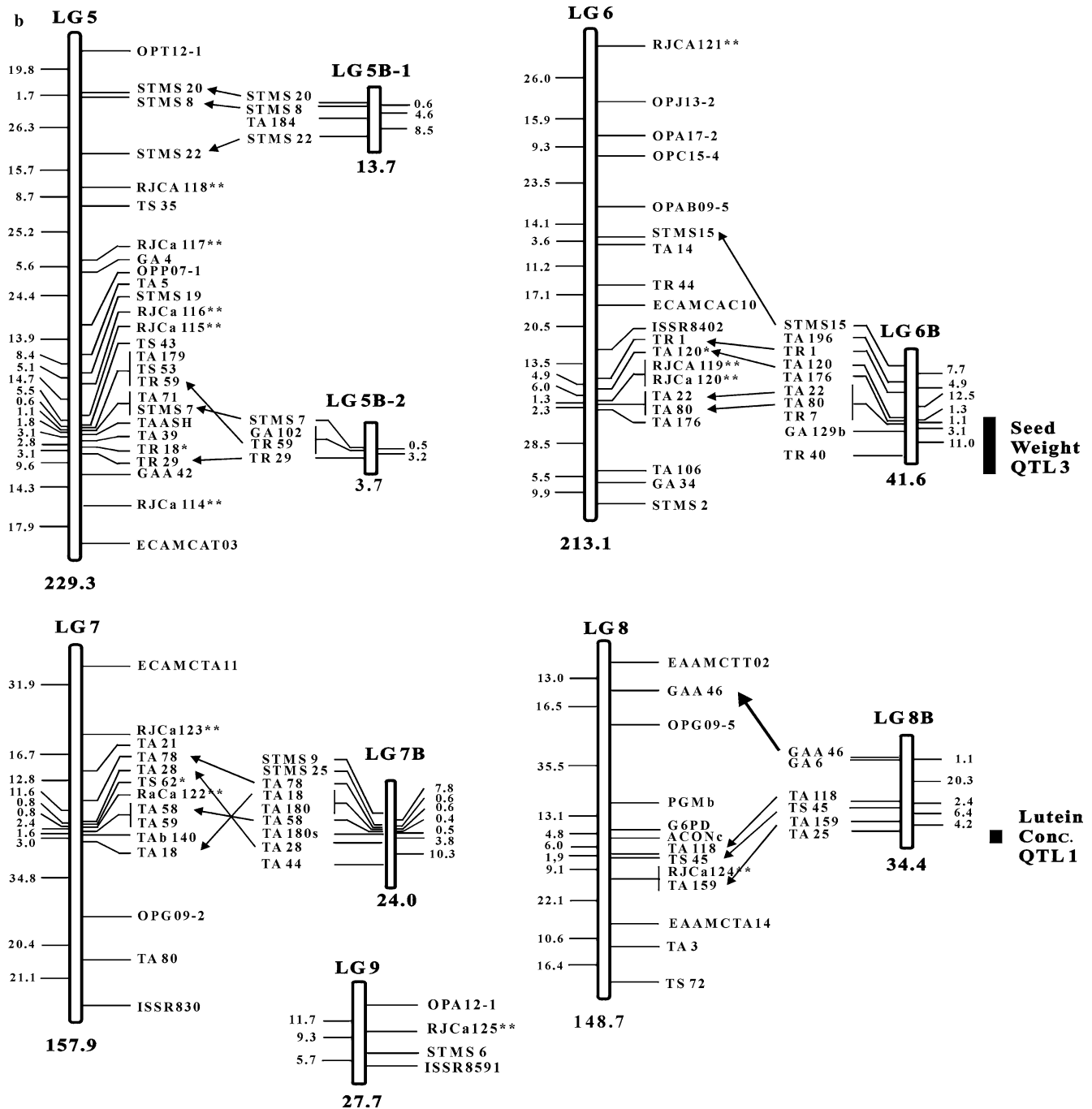
Fig. 3 (Contd.)

interpreted as an indication for partial dominance of cv. *Hadas* alleles that limit seed beta-carotene concentration in this cross.

Both the family variance component and the heritability values observed for seed weight are in good agreement with results from earlier studies on the genetic control of this character in chickpea (Niknejad et al. 1971; Or et al. 1999; Hovav et al. 2003). The relationship between mean seed weight and zeaxanthin concentration had a different pattern (Fig. 1), since about one-half of

the families showed mean zeaxanthin values higher than the mid-parent value (1.93 µg g^{-1} DW). Another feature of the scatterplot of mean zeaxanthin values is that more than 15% of the families had values exceeding that of the high parent (Cr205), while hardly any family had values lower than that of the lower parent (*Hadas*). This may be indicative of either partial dominance of Cr205 alleles promoting zeaxanthin or, alternatively, that the cv. *Hadas* parent carries zeaxanthin-promoting alleles despite its relatively lower concentration.

Table 4 QTLs for beta-carotene and lutein concentration and seed weight detected in this study

Trait	QTL name	Linkage group	LOD score	Indicative markers
beta-Carotene concentration	QTL1	Unlinked	3.9	TS19
	QTL2	3B	3.0	TA64, STMS28
	QTL3	1B	2.1	GA11, TA122
	QTL4	Unlinked	2.1	TR26
Lutein concentration	QTL1	8B	2.4	TA25
Seed weight	QTL1	4B	3.8	GA24, STMS11, GA2
	QTL2	1B	3.2	GA11
	QTL3	6B	2.4	TA120, TR40

Mapping of QTLs for seed weight and carotenoid concentration

Some of the carotenoid QTLs that were declared in the present work had LOD scores lower than 3 (2.1–2.4, in Table 4), and these values should be viewed with caution. Interestingly, however, with respect to the putative location of grain weight genes and loci governing carotenoid levels, our QTL analysis is in good agreement with the genetic correlations (Table 3), suggesting that the detected QTLs are probably true-to-life. For example, we observed a negative correlation of seed weight and beta-carotene concentration in the F_3. This finding is in accordance with the observation of less significant QTLs for seed weight and beta-carotene concentration close to marker GA11 on LG 1B. Since these QTLs– even though they are adjacent to each other—do not overlap, it should be possible to break this linkage using larger segregating populations.

A comparison of the seed weight QTLs on our LG 4B with the mapped seed weight QTLs close to TA130 in an intraspecific population (Cho et al. 2002) shows that the same chromosomal region is involved in determining seed weight in both populations. In our map, the STMS TA130 marker is not closely linked to the seed weight QTL, although it is on the same LG, but is located at quite a distance from it. Also, the linear order markers in our population differs from that of the population of Cho et al. (2002). The reason for this discrepancy may be that all of the markers in the vicinity of TA130 (Fig. 3) showed segregation distortion in our interspecific population, whereas Cho et al. (2002) used an intraspecific population. This implies that the whole region was transmitted as a block to many offspring in our interspecific population. Such a block-transmission is probably not a property of interspecific populations in chickpea per se but specific to our particular population, since in the interspecific population used by Winter et al. (1999, 2000) the same genomic region segregated normally.

Differences in chromosomal linear order (e.g. inversions, translocations) may have contributed to the apparent differences between the present map (especially LGs 3, 4) and other published chickpea maps (e.g. Winter et al. 2000; Cho et al. 2002). Translocations are known to occur between domesticated chickpea and its wild progenitor *C. reticulatum* (Ladizinsky and Adler 1976). However, this interpretation should be considered with caution; first, because the present map is considerably smaller and rather incomplete compared with the standard map of Winter et al. (2000) and, second, because the wild parent used in our study (Cr205) is in fact identical to the wild parent used by Winter et al. (2000) namely, PI489777 (Berger et al. 2003). Hence, further information on the meiotic chromosome configurations in the F_1 cross between cv. *Hadas* and ICC4958 is required to confirm or reject this possibility. Therefore, at the present stage, it is impossible to determine beyond doubt if our seed weight QTL1 represents different loci compared with the seed weight locus identified on LG 4 by Cho et al. (2002).

Since the differences of carotenoid concentration between the two parents involved in the cross are quantitative rather than qualitative (Table 1), the between-family variation probably represents genetic variation in regulatory genes that determine the efficiency of carotenoid bio-synthesis and/or its accumulation in the seeds. This is in contrast to the presence/absence of genes required for carotenoid biosynthesis. This assumption was substantiated by the detection of at least one significant QTL for the concentration of the analysed carotenoids.

Biosynthesis of all carotenoids starts from phytoene and is catalysed by a relatively limited number of enzymes (Cunningham and Gantt 1998; Hirschberg 2001). Coding loci for these enzymes may or may not be located in close vicinity to each other in chickpea. Indeed, we mapped several QTL governing the quantity of carotenoids in seeds. Whether our mapped QTL represent genes coding for enzymes involved in the relevant biosynthetic pathway or regulating factors controlling their quantity in seeds remains to be determined. Similar clustering of QTL for carotenoid concentration was reported for tomatoes (Fulton et al. 2000) and carrot roots (Santos and Simon 2002).

Prospects for breeding for high carotenoid concentration and large seeds in chickpea

Despite the relatively low concentration of carotenoids in the large-seeded cv. *Hadas*, a relatively wide range of beta-carotene and zeaxanthin concentrations (0.1–0.4 μg g^{-1} DW, 1.2–3.2 μg g^{-1} DW, respectively) are present in seeds from cross-progeny of intermediate seed weight (approximately 0.3 g, Fig. 1a, b). This implies that there is considerable scope for improving

(increasing) carotenoid concentration within any genetic background. The generation of such improved large-seeded elite lines with high concentrations of carotenoids may require breeders to break the linkage between the QTL for beta-carotene concentration and seed weight using either other parental lines and/or a larger number of cross-progeny. This strategy could replace the demoting carotenoid alleles in *Hadas* by the promoting alleles from Cr205 or another high-producing donor accession. However, the high costs of chemically analysing large populations by HPLC are a serious drawback for breeding and, consequently, HPLC will probably not become a standard selection tool, even in developed countries. While in some cases pigment concentrations can be detected visually (e.g. tomato high pigment mutations), we were unable to observe colour differences among the mill preparations from the segregating progeny for the HPLC detection. Given the high cost of the HPLC analyses and the limitations to visual selections, molecular markers such as the STMS used in our study could serve as a surrogate selection tool.

Our results also highlight the importance of reliable genetic markers (i.e. STMS) that are inter-population-transferable for genetic analyses. Most of the earlier reported STMS markers (Hüttel et al. 1999; Winter et al. 1999) were polymorphic in the cross used in the present investigation and mapped to the corresponding LGs in the previously used population, thereby allowing a comparison with other studies (Winter et al. 2000; Cho et al. 2002). Therefore, the development of additional STMS markers would benefit and accelerate breeding for the nutritional quality characters of chickpea seeds. As this study shows, the prospects of supplementing the nutritional requirements of under-privileged population sectors in developing countries together with those of an increasing market for "functional" healthy food in the developed world by combining high carotenoid concentration with high weight of chickpeas are promising. Thus, value-added chickpeas and chickpea-based products could expand into old and new markets alike.

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