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Mapping Quantitative Trait Loci for Carotenoid Concentration in Three F_2 Populations of Chickpea

Mohammad K. Rezaei, Amit A. Deokar, Gene Arganosa, Manish Roorkiwal, Sarita K. Pandey, Thomas D. Warkentin, Rajeev K. Varshney, and Bunyamin Tar´an*

M.K. Rezaei, A.A. Deokar, G. Arganosa, T.D. Warkentin, B. Tar'an, Dep. of Plant Sciences, Crop Development Centre, Univ. of Saskatchewan, Saskatoon, Saskatchewan, S7N 5A8, Canada; M. Roorkiwal, S.K. Pandey, R.K. Varshney, ICRISAT, Hyderabad 502324, India.

ABSTRACT Three F_2 populations derived from crosses between cultivars with green and yellow cotyledon colors were used to identify quantitative trait loci (QTLs) associated with carotenoid components in chickpea (*Cicer arietinum* L.) seeds developed by the Crop Development Centre (CDC). Carotenoids including violaxanthin, lutein, zeaxanthin, β-cryptoxanthin, and β-carotene were assessed in the $F_{2:3}$ seeds via high-performance liquid chromatography (HPLC). In the 'CDC Jade' × 'CDC Frontier' population, 1068 bin markers derived from the 50K Axiom CicerSNP array were mapped onto eight linkage groups (LGs). Eight QTLs, including two each for β-carotene and zeaxanthin and one each for total carotenoids, β-cryptoxanthin, β-carotene, and violaxanthin were identified in this population. In the 'CDC Cory' × 'CDC Jade' population, 694 bin markers were mapped onto eight LGs and one partial LG. Quantitative trait loci for β-cryptoxanthin, β-carotene, violaxanthin, lutein, and total carotenoids were identified on LG8. A map with eight LGs was developed from 581 bin markers in the third population derived from the 'ICC4475' × 'CDC Jade' cross. One QTL for β-carotene and four QTLs, one each for β-cryptoxanthin, β-carotene, lutein, and total carotenoids, were identified in this population. The highest phenotypic variation explained by the QTLs was for β-carotene, which ranged from 58 to 70% in all three populations. A major gene for cotyledon color was mapped on LG8 in each population. A significant positive correlation between cotyledon color and carotenoid concentration was observed. Potential candidate genes associated with carotenoid components were obtained and their locations on the chickpea genome are presented.

Abbreviations: CDC, Crop Development Centre; HPLC, high performance liquid chromatography; LG, linkage group; PVE, phenotypic variation explained; QTL, quatitative trait locus; SGR, *stay-green* gene; SNP, single nucleotide polymorphism.

CORE IDEAS

- Quantitative trait locus (QTL) analyses for carotenoids in chickpea were completed for three F_2 populations.
- A moderate number of QTLs and candidate genes associated with carotenoid concentration in chickpea seeds were identified.
- Green cotyledon color is positively associated with provitamin A carotenoids.

Chickpea ($2n = 2x = 16$), with a relatively small genome size (740 Mb; Arumuganathan and Earle, 1991), is the second most important pulse crop globally in terms of production. The combination of protein, carbohydrates, and vitamins, particularly provitamin A carotenoids in seeds, makes chickpea an important component of diets, especially in many developing countries (Abbo et al., 2005; Abu-Salem and Abou-Arab, 2011). Chickpeas with a green or orange cotyledon color tend to have high levels of provitamin A carotenoids, including β-carotene and β-cryptoxanthin (Ashokkumar et al., 2015; Rezaei et al., 2016). The crop is a good model for studying carotenogenesis in legumes because of the availability of its genome sequence (Varshney et al., 2013) and the existence of substantial cotyledon color variation. The expression levels of some genes from the

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*Corresponding author (bunyamin.taran@usask.ca).

carotenoid biosynthesis pathway have been analyzed in chickpea by Rezaei et al. (2016); however, information is lacking on the genes that are responsible for cotyledon color and those involved in the isoprenoid pathway.

The relationships between fruit or root color and the genes in the carotenoid pathway have been reported in tomato (*Solanum lycopersicum* L.) (Liu et al., 2003) and carrot (*Daucus carota* L.) (Just et al., 2009). One approach to identifying the genes associated with carotenoid components is QTL analysis via high-resolution maps (Abbo et al., 2005; Roorkiwal et al., 2017). The availability of a saturated linkage map can increase the power and accuracy in detecting QTLs (Varshney, 2016). Mapping QTLs associated with important carotenoids has been reported in several crops. In a cross between orange and white carrot, two loci, Y and Y2, showed strong association with provitamin A content. Further analysis including fine mapping and transcriptome analysis resulted in the identification of candidate genes responsible for β-carotene content in carrot (Just et al., 2009; Ellison et al., 2017). Quantitative trait locus analysis successfully identified important QTLs for carotenoid components as well as yellow pigment concentration and yellow color index in durum wheat (*Triticum turgidum* L.) (Blanco et al., 2011). Quantitative trait loci for carotenoids and flour color were identified via a set of recombinant inbred lines in wheat (*Triticum aestivum* L.) (Zhao et al., 2013). In cassava (*Manihot esculenta* Crantz), five and three QTLs were responsible for provitamin A content and root color, respectively (Ana et al., 2013). Quantitative trait loci with a strong effect on potato (*Solanum tuberosum* L.) flesh pigmentation were identified on chromosome 5, 8, and 9. A transcript regulator for anthocyanin biosynthesis in potato was colocalized with the QTL for flesh pigmentation on chromosome 9 (Zhang et al., 2009). Candidate genes associated with carotenoid metabolism, including *lycopene epsilon cyclase*, *carotenoid cleavage dioxygenase1*, and *β-carotene hydroxylase*, were mapped in maize (*Zea mays* L.) in an $F_{2:3}$ population (Kandianis et al., 2013). The QTLs identified in the recombinant inbred line population of maize accounted for 4 to 47% of the variation for carotenoid concentration (Jittham et al., 2017). Quantitative trait loci for provitamin A (β-carotene) and lutein content have previously been reported in chickpea (Abbo et al., 2005). Thus far, no report for other carotenoid components in chickpea is available. The main objective of this study was to map the QTLs associated with the concentration of major carotenoid components in three chickpea populations derived from crossing parental lines with different cotyledon colors. The study also evaluated the relationship between cotyledon color and carotenoid levels in each population.

MATERIALS AND METHODS

Plant Materials

Five F_2 populations were initially developed at the CDC at the University of Saskatchewan in 2014. Individual F_2 seeds were sown in 7.6-L pots filled with Sunshine Mix #4 media (SunGrow, Seba Beach, AB, Canada) in the greenhouse at the College of Agriculture and Bioresources in July 2014. The day–night temperature of 22–16°C and a 16-h photoperiod were maintained throughout the growing cycle by artificial light sources. Fertilizer (20–20–20 N–P–K) was added to each pot (3 g L^{-1}) every 2 wk three times after the plants reached 20 cm in height. Watering was done manually two to three times a week depending on the growth stage and was stopped when the plants reached physiological maturity. Individual plants were hand-harvested and the pods were threshed manually.

Inheritance of Cotyledon Color

One hundred F_2 seeds from each population were randomly sampled for cotyledon color scoring. Each seed was cut in half with pliers and the cotyledon color was assessed visually. The goodness of fit of the observed to the expected segregation ratios (three yellow to one green) of cotyledon color in each of the five F_2 populations was assessed via the χ^2 test.

Carotenoid Measurement

Carotenoid measurements were conducted on the $F_{2:3}$ populations derived from crossing parental cultivars with contrasting cotyledon colors and carotenoid content levels. Individual seeds of four randomly selected F_3 seeds derived from each F_2 plant of CDC Jade × CDC Frontier, CDC Cory × CDC Jade and ICC4475 × CDC Jade populations were used for carotenoid analysis via a HPLC assay. The concentrations of five carotenoid components including violaxanthin, lutein, zeaxanthin, β-cryptoxanthin, and β-carotene were measured. Carotenoid extraction and the HPLC assay procedure were carried out according to the method described by Rezaei et al. (2016).

Statistical Analysis

Tests of the goodness of fit of cotyledon color segregation; means comparison for carotenoid concentrations in green, segregating, and yellow cotyledon color groups within each population; and the correlation analysis were performed with SAS version 9.3 (SAS Institute Inc., Cary, NC).

DNA Extraction and Genotyping

The total DNA from leaves of individual F_2 plants from the CDC Jade × CDC Frontier, CDC Cory × CDC Jade, and ICC4475 × CDC Jade populations was extracted following the protocol of the DNeasy Plant Mini Kit (Qiagen, Germantown, MD) in the Pulse Crop Genetics Lab at the Department of Plant Sciences, University of Saskatchewan. After checking the quality and quantity of the DNA, the samples were sent to ICRISAT, Hyderabad, Patancheru, India, for genotyping with the 50K Axiom CicerSNP array (Roorkiwal et al., 2017).

Map Construction and QTL Analysis

Each single nucleotide polymorphism (SNP) locus was tested against the expected segregation ratio of 1:2:1 in a F_2 population using the χ^2 test in MapDisto version

Table 1. χ^2 Test of the goodness of fit of the observed to the expected segregation ratios (3:1, yellow vs. green) of cotyledon color in five F_2 populations derived from crosses of cultivars with contrasting cotyledon color.

Parent 1†	Parent 2	Yellow cotyledons	Green cotyledons	χ^2-value‡	*P*-value
CDC Verano	CDC 441–34	74	26	0.053	0.81
CDC Verano	CDC Frontier	70	30	1.33	0.24
CDC Jade	CDC Frontier	70	30	1.33	0.24
CDC Cory	CDC Jade	75	25	0.005	1.00
ICC4475	CDC Jade	78	22	0.37	0.48

† CDC Verano, *kabuli*, green cotyledon; CDC 441–34, *kabuli*, yellow cotyledon; CDC Frontier, *kabuli*, yellow cotyledon; CDC Jade, *desi*, green cotyledon; CDC Cory, *desi*, yellow cotyledon; ICC4475, *desi*, yellow cotyledon and black seed coat.

‡ $df = 1$, $\chi^2(0.05) = 3.841$, $\chi^2(0.01) = 6.635$.

2.0 software (Heffelfinger et al., 2017). The SNPs with significant deviation ($P \leq 0.05$) from the expected ratio and loci with more than 10% missing data were removed from further analysis.

The cotyledon color trait was directly integrated into the genetic map along with the SNP markers. In the case of CDC Jade × CDC Frontier, CDC Jade, with a green cotyledon color, was used as the female parent and CDC Frontier, with yellow cotyledons, as the male parent; progeny with green, yellow, and segregating cotyledon color were converted to A, B, and H genotypes, respectively . The opposite was done for the CDC Cory × CDC Jade and ICC4475 × CDC Jade populations since CDC Jade was used as the male parent and the yellow cotyledon cultivars as the female parent. Quantitative trait locus analysis was conducted with QTL IciMapping version 3.3 software (Meng et al., 2015). The inclusive composite interval mapping approach based on bin markers was used for mapping QTLs through the ICIM-ADD option in the IciMapping software. One thousand permutations were done to set the logarithm of the odds threshold with a type I error rate of ≤0.05.

RESULTS

Inheritance Study

The χ^2 analysis confirmed that cotyledon color is controlled by a single gene, in which yellow cotyledon color is dominant over green cotyledon color (Table 1).

Carotenoid Measurement

Among the four parental cultivars, CDC Jade had the highest concentration of provitamin A (combined β-carotene and β-cryptoxanthin) and total carotenoids (3.0 and 40.0 μg g^{-1}, respectively). The concentrations of provitamin A and total carotenoids in other parents were as follows: 0.0 and 10.6 μg g^{-1} in CDC Frontier, 0.1 and 11.1 μg g^{-1} in CDC Cory, and 0.0 and 12.3 μg g^{-1} in ICC4475, respectively (Table 2). The seeds of $F_{2:3}$ families from each population were divided into three groups based on cotyledon color as follows: yellow, green, and segregating. In the CDC Jade × CDC Frontier population, 40 families were yellow, 20 were green, and 58 were segregating. In the CDC Cory × CDC Jade population, 37 families were yellow, 19 were green, and 64 were segregating. In the ICC 4475 × CDC Jade population, 42 families were yellow, 21 were green, and 55 were segregating (Supplemental File S1). The means comparison of total carotenoid concentrations from the three groups showed that the green cotyledon group had a significantly higher carotenoid concentration than the yellow group, whereas the segregating individuals were in between the green and yellow groups in CDC Jade × CDC Frontier population. In the CDC Cory × CDC Jade population, the green group was in between the plants with segregating and yellow cotyledon colors. In the ICC4475 × CDC Jade population, both green and segregating progeny groups were in the same group that are significantly higher than the yellow cotyledon genotypes (Supplemental File S1). The range of the concentrations of violaxanthin, lutein, zeaxanthin, β-carotene, β-cryptoxanthin, and total carotenoids in the yellow, segregating, and green cotyledon groups showed that the largest variations in the different carotenoid components were in the yellow cotyledon group across all three populations. The segregating and green cotyledon groups had close ranges for different carotenoid concentrations across the three populations (Table 3).

Table 2. The mean concentration (μg g^{-1}) and SD of seed carotenoids, including violaxanthin, lutein, zeaxanthin, β-cryptoxanthin, β-carotene, and total carotenoids measured by high-performance liquid chromatography in four chickpea parents: CDC Frontier, CDC Cory, ICC4475, and CDC Jade.

	CDC Frontier		CDC Cory		ICC4475		CDC Jade	
Component	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Violaxanthin	0.09	0.67	0.51	1.2	0.64	0.57	3.04	0.89
Lutein	8.76	0.23	9.28	0.56	10.66	0.38	31.4	0.76
Zeaxanthin	1.75	0.28	1.22	0.76	1.03	0.59	3	0.34
β-Cryptoxanthin	0	–	0	–	0	–	0	–
β-carotene	0	–	0.1	0.44	0	–	2.56	0.53
Total carotenoids	10.6	0.98	11.11	1.69	12.33	1.02	40	1.71

The results of the carotenoid analysis of each family from the three populations are presented in Supplemental File S2. Transgressive segregants were observed in the $F_{2:3}$ families for each carotenoid component (Supplemental File S3); in the case of lutein and zeaxanthin, several $F_{2:3}$ families had concentrations exceeding the concentration of the parents. The highest concentrations for provitamin A and total carotenoids (13 and 90 μg g^{-1}, respectively) were observed in the ICC4475 × CDC Jade population (Supplemental File S3).

Correlation Analysis

In the CDC Jade × CDC Frontier population, violaxanthin concentration was positively correlated with lutein ($r = 0.72$, $P < 0.001$), zeaxanthin ($r = 0.25$, $P < 0.01$), β-cryptoxanthin ($r = 0.49$, $P < 0.001$), β-carotene ($r = 0.70$, $P < 0.001$), and total carotenoids ($r = 0.81$, $P < 0.001$). Lutein showed a significant positive correlation with zeaxanthin ($r = 0.52$, $P < 0.01$), β-cryptoxanthin

Table 3. the range of concentration of the carotenoid components in yellow, segregating, and green cotyledon classes in three F_2 populations including CDC Jade × CDC Frontier, CDC Cory × CDC Jade, and ICC4475 × CDC Jade.

Component	Cotyledon color	CDC Jade × CDC Frontier			CDC Cory × CDC Jade			ICC4475 × CDC Jade		
		Range	Mean	SD	Range	Mean	SD	Range	Mean	SD
Violaxanthin	Yellow	0–6.4	1.38	0.83	0.43–2.95	1.44	0.56	0.46–3.2	1.5	0.65
	Segregating	0.46–4.21	2.27	0.85	1.39–4.01	2.45	0.59	1.16–4.3	2.36	0.69
	Green	1.06–5.2	3.36	1.41	1.14–5.19	2.19	1.06	1.22–7.73	2.83	1.99
Lutein	Yellow	14.28–45.46	24.66	6.44	1.26–60.1	34.31	13.26	19.02–54–48	37.02	9.14
	Segregating	15.3–37.85	26.42	5.88	31.32–63.23	45.23	6.64	34.64–56.13	45.12	5.14
	Green	22.54–35.37	26.64	3.88	27.28–52.31	37	7.1	28.63–59.56	38.09	7.93
Zeaxanthin	Yellow	0.66–7.77	4.46	1.99	0.27–8.35	5.23	2.15	2.07–8.18	4.58	1.51
	Segregating	1.48–6.87	4.42	1.2	2.28–7.75	4.98	0.97	2.05–6.56	4.39	1.01
	Green	2.86–5.62	3.89	1.08	2.41–8.48	4.56	1.48	0.01–7.22	4.23	1.46
β-Cryptoxanthin	Yellow	0–0.9	0.02	0.08	0–0.76	0.08	0.18	0–1.42	0.23	0.43
	Segregating	0–1.2	0.2	0.29	0–1.33	0.45	0.34	0–1.19	0.27	0.33
	Green	0–2.73	0.55	0.73	0–3.93	1.39	0.95	0–5	1.71	1.38
β-Carotene	Yellow	0–3.04	0.32	0.48	0–2.29	0.71	0.68	0–3.12	0.98	0.76
	Segregating	0–2.09	1.04	0.58	0–3.51	1.88	0.63	0.38–4.2	2.27	0.86
	Green	2.19–3.84	2.6	0.6	2.4–6.28	4.06	1.08	2.63–7.47	4.3	1.2
Total carotenoids	Yellow	14.94–58.14	30.84	8.65	1.96–70.44	41.79	15.9	21.55–64.97	44.42	10.86
	Segregating	18.46–49.61	34.35	7.79	40.91–77.63	55.01	7.61	40.7–66.97	54.42	6.25
	Green	28.68–51.54	37.04	6.55	35.42–67.47	49.28	9.01	38.01–83.74	51.18	10.81

($r = 0.23$, $P < 0.05$), β-carotene ($r = 0.44$, $P < 0.001$), and total carotenoids ($r = 0.97$, $P < 0.001$). Zeaxanthin concentration was positively correlated only with the total carotenoids ($r = 0.60$, $P < 0.001$). β-Cryptoxanthin was positively correlated with β-carotene ($r = 0.51$, $P < 0.001$) and total carotenoids ($r = 0.36$, $P < 0.001$). Moreover, β-carotene showed a positive correlation with total carotenoids ($r = 0.56$, $P < 0.001$; Table 4).

In the second population (CDC Cory × CDC Jade), violaxanthin concentration showed positive correlations with lutein ($r = 0.78$, $P < 0.001$), β-carotene ($r = 0.39$, $P < 0.001$), and total carotenoids ($r = 0.79$, $P < 0.001$). Lutein showed significant positive correlations with zeaxanthin ($r = 0.51$, $P < 0.01$), β-carotene ($r = 0.23$, $P < 0.05$), and total carotenoids ($r = 0.98$, $P < 0.001$). Similar to the first population, zeaxanthin concentration was positively correlated with total carotenoids ($r = 0.57$, $P < 0.001$). β-Cryptoxanthin showed positive correlations with β-carotene ($r = 0.74$, $P < 0.001$) and total carotenoids ($r = 0.18$, $P < 0.05$). In addition, β-carotene was positively correlated with total carotenoids ($r = 0.36$, $P < 0.001$; Table 4).

In the third population (ICC 4475 × CDC Jade), violaxanthin concentration showed positive correlations with lutein ($r = 0.56$, $P < 0.001$), β-carotene ($r = 0.62$, $P < 0.001$), and total carotenoids ($r = 0.65$, $P < 0.001$). Lutein showed significant positive correlations with zeaxanthin ($r = 0.29$, $P < 0.01$), β-carotene ($r = 0.37$, $P < 0.001$), and total carotenoids ($r = 0.96$, $P < 0.001$). Similar to the first and second populations, zeaxanthin concentration was only positively correlated with total carotenoids ($r = 0.36$, $P < 0.001$). β-Carotene showed positive correlations with β-cryptoxanthin ($r = 0.50$, $P < 0.001$) and with total carotenoids ($r = 0.55$, $P < 0.001$; Table 4).

Linkage Map Development and QTL Analysis in Three F_2 Populations

The genotypic data generated by the 50K Axiom CicerSNP array were examined for missing data and segregation patterns. The SNP markers that did not fit the expected 1:2:1 ratio, as well as the markers with more than 10% missing data, were removed from further analysis. After this filtering, 1068 bins (4296 SNPs) were selected for linkage mapping in the CDC Jade × CDC Frontier population (indicated by the label JF). The markers were distributed over eight LGs, in which the highest (231) and the lowest (62) number of markers were found in LG1 and LG2, respectively (Table 5). The average distance between two markers across all LGs was 3.01 cM. Three QTLs were identified on LG1: one each for zeaxanthin (qZea-1-JF), β-carotene (q-Crt-1-JF), and lutein concentration (q-Lut-1-JF). An additional QTL was found on LG5 for zeaxanthin (q-Zea-5-JF). Four QTLs were identified on LG8: one each for total carotenoids (q-Tot-8-JF), β-cryptoxanthin (q-Cryp-8-JF), β-carotene (q-Crt-8-JF), and violaxanthin (q-Vio-8-JF). The q-Crt-8-JF and q-Crt-1-JF QTLs explained the highest (68%) and the lowest (5%) proportion of phenotypic variation, respectively, in this population. The cotyledon color (CotCol) trait was mapped on LG8 overlapping with q-Crt-8-JF and q-Vio-8-JF (Table 6; Fig. 1A).

In the CDC Cory × CDC Jade population (indicated by the label CJ), 694 bins (2541 SNP markers) were mapped onto eight LGs and one partial LG. The maximum number of SNP markers (216) was placed in LG4 and the minimum number (38) was placed in LG3 (Table 5). The average distance between two markers across all LGs was 2.85 cM. Three major QTLs were found on LG 8. One QTL each was identified for β-cryptoxanthin (q-Cryp-8-CJ) and β-carotene (q-Crt-8-CJ). Another genomic region on LG8

Table 4. Pairwise Pearson correlation coefficients for carotenoid components including violaxanthin, lutein, zeaxanthin, β-cryptoxanthin, β-carotene, and total carotenoid concentration (μg g^{-1}) in three F_2 populations.

Component	Lutein	Zeaxanthin	β-Cryptoxanthin	β-Carotene	Total†
CDC Jade × CDC Frontier					
Violaxanthin	0.72***	0.25**	0.49***	0.70***	0.81***
Lutein	–	0.52***	0.23*	0.44***	0.97***
Zeaxanthin	–	–	0.09ns	0.04ns	0.60***
β-Cryptoxanthin	–	–	–	0.51***	0.36***
β-Carotene	–	–	–	–	0.56***
CDC Cory × CDC Jade					
Violaxanthin	0.78***	0.13ns‡	0.18ns	0.39***	0.79***
Lutein	–	0.51***	0.04ns	0.23*	0.98***
Zeaxanthin	–	–	0.09ns	–0.01ns	0.57***
β-Cryptoxanthin	–	–	–	0.74***	0.18*
β-Carotene	–	–	–	–	0.36***
ICC 4475 × CDC Jade					
Violaxanthin	0.56***	–0.12ns	0.07ns	0.62***	0.65***
Lutein	–	0.29**	–0.12ns	0.37***	0.96***
Zeaxanthin	–	–	0.16ns	–0.07ns	0.36***
β-Cryptoxanthin	–	–	–	0.50***	0.09ns
β-Carotene	–	–	–	–	0.55***

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.001 probability level.

† Total carotenoid concentration in three populations.

‡ ns, nonsignificant.

Table 5. Summary of linkage maps of three chickpea F_2 populations including CDC Jade × CDC Frontier (JF), CDC Cory × CDC Jade (CJ), and ICC 4475 × CDC Jade (IJ).

Population	LG†	No. of bin markers per LG	LG length	Average distance of loci
			cM	
JF	1	231	714.01	3.09
	2	62	217.45	3.5
	3	143	418.87	2.92
	4	125	453.76	3.63
	5	106	382.77	3.61
	6	194	456.41	2.35
	7	114	371.19	3.25
	8	93	207.71	2.23
CJ	1	61	266.47	4.36
	2	85	230.93	2.71
	3	38	158.05	4.15
	4	216	500.84	2.31
	5	56	118.94	2.12
	6	115	310.17	2.69
	7	84	275.57	3.28
	8	39	122.96	3.15
IJ	1	11	124.42	11.31
	2	94	269.18	2.86
	3	89	324.95	3.65
	4	166	461.57	2.78
	5	77	272.35	3.53
	6	59	235.32	3.98
	7	47	219.45	4.66
	8	38	162.73	4.28

† LG, linkage group.

was associated with three QTLs (q-Vio-8-CJ, q-Lut-8-CJ, and q-Tot-8-CJ) for violaxanthin, lutein, and total carotenoids, respectively. Each QTL accounted for a moderate to large amount of phenotypic variation in this population. The highest and the lowest phenotypic variation explained (PVE) was 70 and 24% for β-carotene and total carotenoids, respectively (Table 6; Fig. 1B).

In addition to the CotCol marker, the previously reported *stay-green* gene (*SGR*) was identified in the CDC Jade × CDC Frontier and CDC Cory × CDC Jade populations on LG8 (2,047,217–2,049,321 bp) within the QTLs for violaxanthin (Vio-8-JF), β-carotene (q-Crt-8-JF), β-cryptoxanthin (q-Cryp-8-JF), and total carotenoids (q-Tot-8-JF) in the CDC Jade × CDC Frontier population and QTLs for violaxanthin (Vio-8-CJ), lutein (Lut-8-CJ), β-carotene (q-Crt-8-CJ), and total carotenoids (q-Tot-8-CJ) in the CDC Cory × CDC Jade population.

In the ICC4475 × CDC Jade population (indicated by the label IJ), 581 bins (2550 SNP markers) were used for linkage mapping, which resulted in eight LGs. The highest and lowest numbers of markers were observed in LG4 (166 markers) and LG1 (11 markers), respectively (Table 5). The average distance between two markers across all LGs was 3.56 cM. One QTL for β-carotene (q-Crt-8-IJ) was found on LG3 that explained 9% of the phenotypic variation for β-carotene concentration. Four QTLs were found on LG8: one each for β-cryptoxanthin (q-Cryp-8-IJ), β-carotene (q-Crt-8-IJ), lutein (q-Lut-8-IJ), and total carotenoids (q-Tot-8-IJ). The highest PVE was found for β-carotene (58%), which overlapped with cotyledon color (Table 6, Fig. 1C). The *pyruvate decarboxylase* and *SGR* genes are the most important genes with functions related to carotenoid concentration that are located within the QTLs in the CDC Jade × CDC Frontier and CDC Cory × CDC Jade populations (Table 7). The genetic and physical locations of each SNP marker in each population are presented in Supplemental File S4.

DISCUSSION

Three F_2 populations derived by crossing cultivars with different cotyledon and seed coat colors were evaluated for carotenoids. CDC Jade was used in each cross as it had higher carotenoid concentrations than other cultivars, in addition to its distinct green cotyledon color (Rezaei et al., 2016).

The χ^2-analysis for cotyledon color segregation across the five F_2 populations showed the ratio of three yellow and one green was the best fit for each population. The results confirmed that cotyledon color is controlled by a single gene, with yellow cotyledon being dominant over green cotyledon. The same result was previously reported in pea (*Pisum sativum* L.) (Mendel, 1866; Sato et al., 2007). However, seed coat color in chickpea is more

Table 6. Results of quantitative trait locus QTL) analysis in three F_2 populations of chickpea, showing the number of QTLs, traits, the chromosomal location of each QTL, map position, closest marker, bordering markers, logarithm of the odds (LOD) values, phenotypic variance explained (PVE), additive, dominance, and confidence interval (CI) are shown.

QTL	Carotenoid component	Chr†	Position	Closest marker	Left marker	Right marker	LOD value	PVE	Additive‡	Dominance‡	95%CI
			cM					%			cM
CDC Jade × CDC Frontier											
q-Zea-1-JF	Zeaxanthin	1	325	AX-123644659	AX-123618241	AX-123618287	4	14	0.6	0.45	323.5–327.5
q-Crt-1-JF	β-carotene	1	554	AX-123641029	AX-123617179	AX-123617173	4.1	5	0.28	–0.17	547.5–558.5
q-Lut-1-JF	Lutein	1	618	AX-123638575	AX-123615858	AX-123615414	5.9	22	2.71	–2.91	611.5–625.5
q-Zea-5-JF	Zeaxanthin	5	367	AX-123632228	AX-123640067	AX-123662533	5.8	20	–0.8	–0.47	364.5–369.5
q-Tot-8-JF	Total	8	168	AX-123657409	AX-123642874	AX-123657408	4	13	3.75	0.74	166.5–180.5
q-Cryp-8-JF	β-cryptoxanthin	8	176	AX-123637790	AX-123657409	CotCol	5.5	22	0.26	–0.08	172.5–180.5
q-Crt-8-JF	β-carotene	8	177	AX-123657409	CotCol	AX-123637790	34.5	68	1.17	0	176.5–179.5
q-Vio-8-JF	Violazanthin	8	180	AX-123657409	CotCol	AX-123637790	11.8	37	1.03	–0.25	176.5–183.5
CDC Cory × CDC Jade											
q-Cryp-8-CJ	β-cryptoxanthin	8	97	AX-123642874	AX-123657408	AX-123637792	17.2	46	–0.65	0.031	95.5–97.5
q-Crt-8-CJ	β-carotene	8	99	AX-123642869	AX-123637792	CotCol	31	70	–1.69	–0.44	97.5–100.5
q-Vio-8-CJ	Violaxanthin	8	101	AX-123637792	CotCol	AX-123642869	9.2	34	–0.37	0.63	98.5–103.5
q-Lut-8-CJ	Lutein	8	101	AX-123637792	CotCol	AX-123642869	8.3	25	–1.64	9.89	100.5–103.5
q-Tot-8-CJ	Total	8	101	AX-123637792	CotCol	AX-123642869	8.2	24	–3.53	10.77	100.5–103.5
ICC 4475 × CDC Jade											
q-Crt-3-IJ	β-carotene	3	201	AX-123646778	AX-123659050	AX-123619520	6.1	9	0.62	–0.2	199.5–203.5
q-Cryp-8-IJ	β-cryptoxanthin	8	20	AX-123642855	AX-123642846	CotCol	17.4	41	–1.05	–1.05	10.5–26.5
q-Crt-8-IJ	β-carotene	8	29	AX-123642855	AX-123642846	CotCol	24.5	58	–1.59	–0.41	25.5–29.5
q-Lut-8-IJ	Lutein	8	30	AX-123637737	CotCol	AX-123642855	7.6	25	–0.03	8.16	17.5–35.5
q-Tot-8-IJ	Total	8	30	AX-123637737	CotCol	AX-123642855	6.3	22	–2.7	7.38	20.5–34.5

† Chr, chromosome.

‡ Positive or negative additive and dominance effects indicated increased effects contributed by alleles from the female parent (CDC Jade in CDC Jade × CDC Fronter; CDC Cory in CDC Cory × CDC Jade; ICC4475 in ICC4475 × CDC Jade) or from the male parent (CDC Frontier in CDC Jade × CDC Fronter; CDC Jade in CDC Cory × CDC Jade and ICC4475 × CDC Jade), respectively.

diverse than cotyledon color and at least three genes control this trait (Meena et al., 2004).

The use of the 50K Axiom CicerSNP Array on the three F_2 populations allowed the development of relatively dense linkage maps and the identification of important QTLs associated with various carotenoid components. The average distances between two markers in the linkage map of the CDC Jade × CDC Frontier and CDC Cory × CDC Jade populations were relatively similar but in the ICC4475 × CDC Jade population, the distance was slightly larger. The relatively larger marker distance in the ICC4475 × CDC Jade population is the consequence of the lower number of markers used for map construction than in the other two populations. The smaller number of bins resulted in lower map resolution (Jittham et al., 2017). Furthermore, the confidence interval of three out of five QTLs in the ICC4475 × CDC Jade population was larger than 10 cM.

Eight LGs were constructed in each population. In the first linkage map for the CDC Jade × CDC Frontier population, LG1 and LG8 were the largest (714.01 cM) and shortest (207.71 cM) LGs, respectively (Table 5). In the second linkage map for the CDC Cory × CDC Jade population, the largest and shortest LGs were LG4 (500.84 cM) and LG5 (118.94 cM). Linakge Group 4 at 461.57 cM and LG1 at 124.42 cM were the largest and shortest LGs in the cross between ICC 4475 and CDC Jade. The map coverage in the three populations was as follows: 3222. 17 cM in CDC Jade × CDC Frontier, 1983.93 cM in CDC Cory × CDC Jade, and 2069.97 cM in ICC 4475 × CDC Jade (Table 5). The map coverage in all three populations was higher than the coverage in five crosses between *desi* and *kabuli* chickpeas (426.99 cM) in a previous report by Millan et al. (2010). The differences could be because of the higher number of markers used in the current study and the difference in the population sizes.

In the CDC Jade × CDC Frontier population, at least one QTL for the concentration of each carotenoid component was identified. This population, in particular, captured the most genomic regions linked to carotenoids. This population had the most contrasting parents, as CDC Jade had the highest carotenoid concentration, whereas CDC Frontier had the lowest carotenoid concentration. This condition made this population ideal for mapping QTLs for carotenoids. In addition, the number of bins used for linkage mapping of this population was almost double than that of other crosses. The identification of the largest number of QTLs in this population is a result of high genetic variation in the progeny.

The green cotyledon color locus (*CotCol*) was mapped to LG8 in each population. The usefulness of cotyledon color as a morphological marker was demonstrated in field pea and faba bean (*Vicia faba* L.) (Mendel, 1866; Cruz-Izquierdo et al., 2012). The majority of the

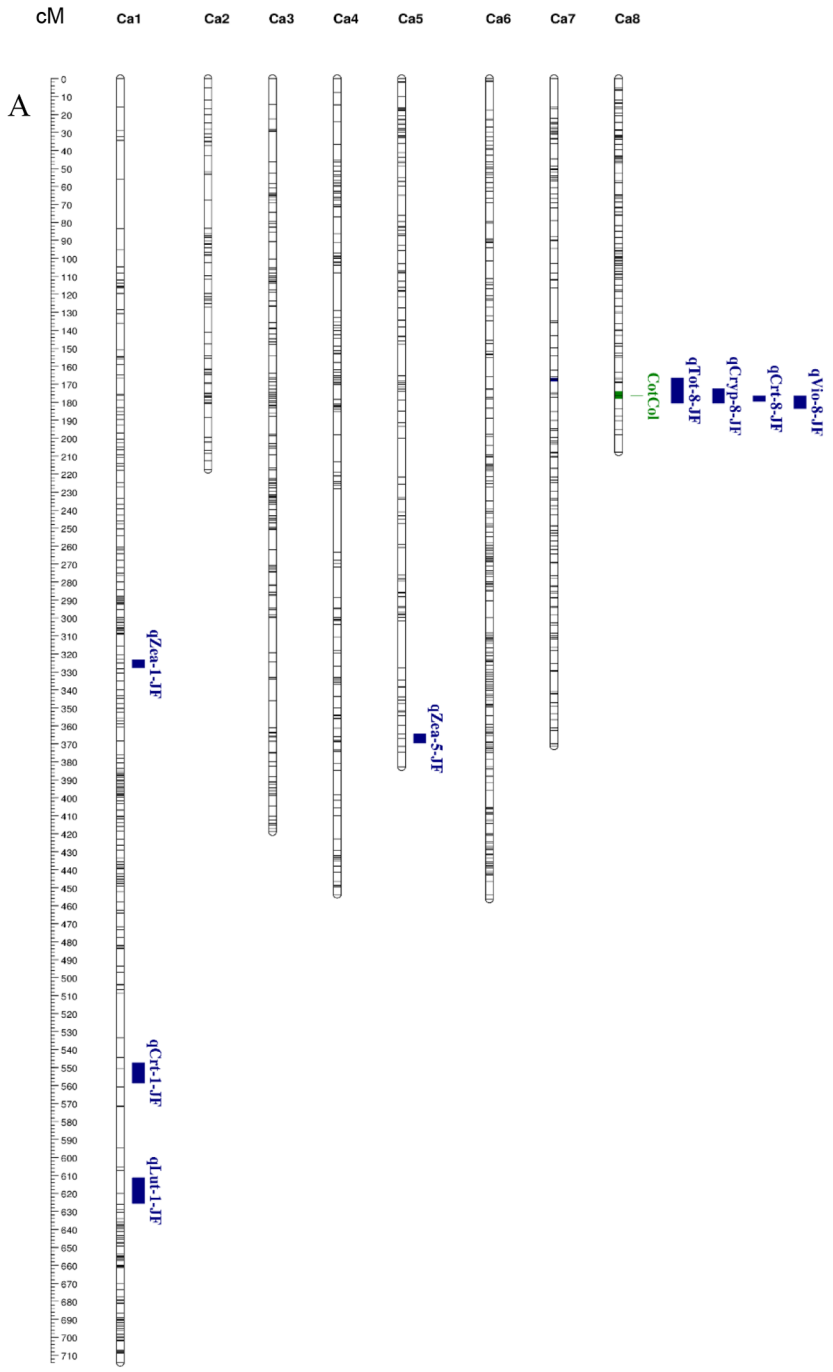

Fig. 1. (A) Genetic map of the F_2 populations of chickpea from a cross between CDC Jade and CDC Frontier showing the quantitative trait loci (QTLs) associated with carotenoids on Linkage Groups (LGs) 1 (β-carotene, zeaxanthin, and lutein), 5 (zeaxanthin), and 8 (violaxanthin, β-carotene, β-cryptoxanthin, and total carotenoids). The cotyledon color marker (*CotCol*) was mapped on LG8 in all three populations. Map unit is cM.

QTLs for different carotenoids were located in the vicinity of the *CotCol* locus on the map.

In maize and wheat, the genes responsible for carotenoid biosynthesis are distributed across the genome (Owens et al., 2014; Colasuonno et al., 2017). The same results have been observed in chickpea. However, in our study we found that the QTLs for different carotenoids were in clusters. This result is in agreement with the results obtained by Santos and Simon (2002) in carrot. According to the correlation analysis, total carotenoid level was significantly correlated with all components in the three populations, except for β-cryptoxanthin in the cross between ICC 4475 and CDC Jade. Within the QTL cluster on LG8, the QTL for total carotenoids was common in all three populations. It is likely that the specific region on LG8 has a key role in controlling carotenoid biosynthesis in chickpea. The clustering of QTLs for carotenoids is an evolutionary event that needs further investigation (Santos and Simon, 2002). The exact positions of the common QTLs varied slightly among the

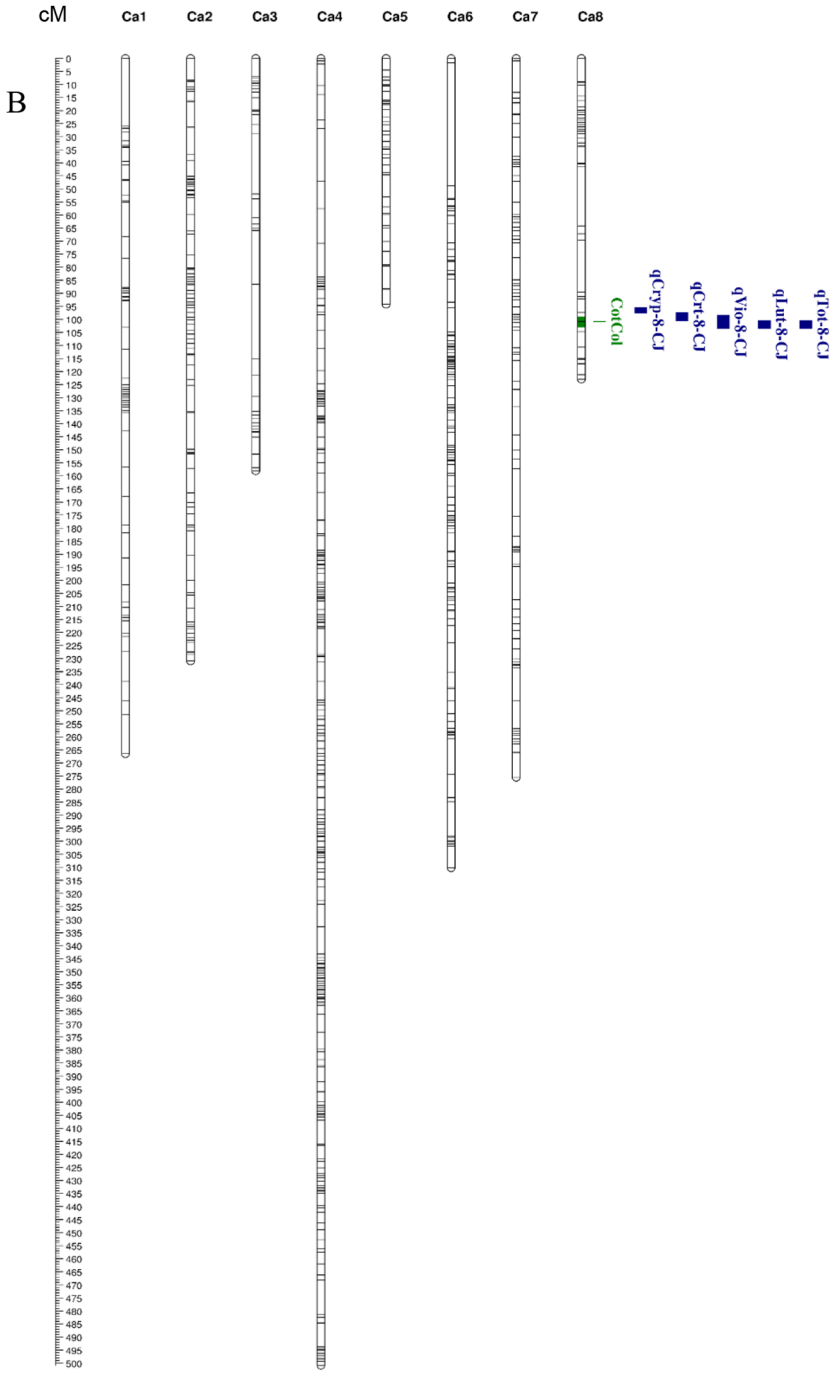

Fig. 1. (B) Genetic map of the F_2 population from a cross between CDC Cory and CDC Jade showing the QTLs for β-cryptoxanthin, violaxanthin, β-carotene, lutein, and total carotenoids on LG8. The cotyledon color marker (*CotCol*) was mapped on LG8 in all three populations. Map unit is cM.

three populations, probably because of the differences in map density among the populations (Long et al., 2008).

The *SGR* gene was found within the QTLs for violaxanthin (Vio-8-JF), β-carotene (q-Crt-8-JF), β-cryptoxanthin (q-Cryp-8-JF), and total carotenoids (q-Tot-8-JF) next to the *CotCol* locus in the CDC Jade × CDC Frontier population. The *SGR* gene is responsible for chlorophyll breakdown. Mutation in this gene leads to the stay-green phenotype (Ren et al., 2007; Sato et al., 2007). Pea plants containing the mutant allele (*sgr*) produced green seeds and their leaves retained their greenness during senescence (Barry et al., 2008). We observed the same situation in the green cotyledon chickpea cultivars CDC Jade and 'CDC Verano'. Rezaei et al. (2016) confirmed that the carotenoid concentration decreased as the seeds matured but in green cotyledon cultivars, the reduction rate was slower than in the yellow cotyledon chickpea cultivars. In environments with water scarcity, higher yield was observed in genotypes with stay-green grain than in the yellow one (Thomas and Howarth, 2000). The high stability of the light harvesting complex (Sato et al., 2007) and low cleavage and hydroxylation

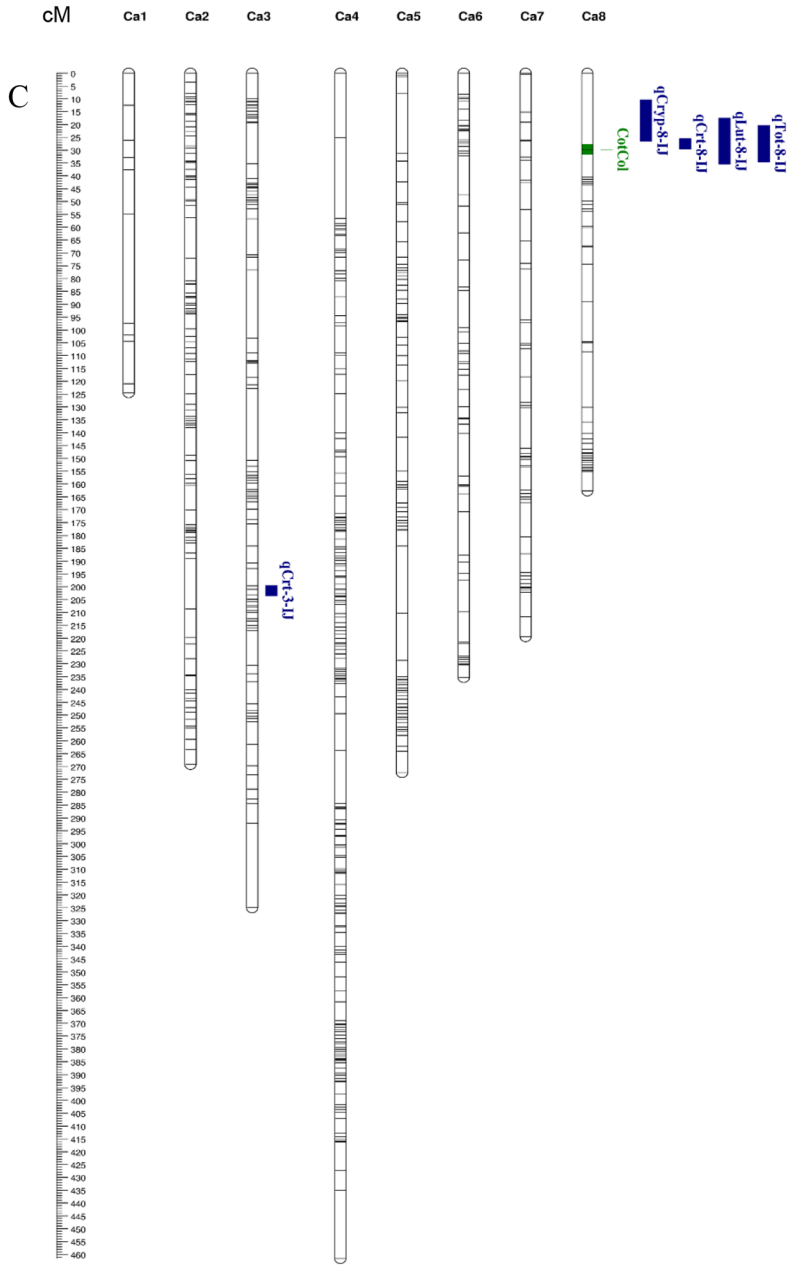

Fig. 1. (C) Genetic map of the F_2 population from a cross between ICC 4475 and CDC Jade showing the QTLs for β-carotene, β-cryptoxanthin, lutein, and total carotenoids on LG3 and LG8. The cotyledon color marker (*CotCol*) was mapped on LG8 in all three populations. Map unit is cM.

activity (Rezaei et al., 2016) were responsible for the elevated carotenoid concentration in the green cotyledon chickpeas. Moreover, the *pyruvate decarboxylase 1* gene, which is located within the QTL for lutein (q-Lut-1-JF) in the CDC Jade × CDC Frontier population, is involved in the biosynthesis of 1-deoxy-D-xylulose 5-phosphate (Rodriguez-Concepcion and Boronat, 2002). The methylerythritol 4-phosphate pathway produces isopentenyl pyrophosphate from 1-deoxy-D-xylulose 5-phosphate and is finally converted to geranylgeranyl pyrophosphate, which is the precursor for carotenoids (Giuliano, 2014). It has been reported that the expression of *pyruvate decarboxylase 1* is positively correlated with β-carotene levels in orange melon (*Cucumis melo* L.) Yuan et al., 2015).

In the CDC Cory × CDC Jade population, four QTLs for violaxanthin (Vio-8-CJ), lutein (Lut-8-CJ), β-carotene (q-Crt-8-CJ), and total carotenoids (q-Tot-8-CJ) colocalized with *SGR*. It is important to note that lutein and β-carotene are common carotenoids in the photosystem of green tissues. The absence of chlorophyll breakdown, which results in green color, elevated provitamin A concentrations in these populations.

The highest PVE in all three populations was obtained from the QTL for β-carotene concentrations as follows: q-Crt-8-CJ (69% PVE), q-Crt-8-JF (67% PVE), and q-Crt-8-IJ (58% PVE). This QTL is present in all three populations and is closely linked to *CotCol*. It showed that a common genomic region is associated with

Table 7. Two candidate genes for carotenoid biosynthesis and concentration located within the quantitative trait loci (QTLs) for different carotenoid components in two populations of chickpea [CDC Jade × CDC Frontier (JF) and CDC Cory × CDC Jade (CJ)], showing the gene ID, chromosome (Chr), physical location in bp, and the putative function of the candidate genes.

Carotenoid component	QTL	Gene ID	Chr (location in bp)	Putative function
CDC Jade × CDC Frontier				
Lutein	q-Lut-1-JF	*LOC101493012*	Chr 1 (1,181,643–1,187,430)	Pyruvate decarboxylase 1-like
β-cryptoxanthin	q-Cryp-8-JF	*LOC101509366*	Chr 8 (2,047,214–2,049,321)	Protein stay-gGreen chloroplastic-like
β-carotene	q-Crt-8-JF	*LOC101509366*	Chr 8 (2,047,214–2,049,321)	Protein stay-green chloroplastic-like
Violaxanthin	q-Vio-8-JF	*LOC101509366*	Chr 8 (2,047,214–2,049,321)	Protein stay-green chloroplastic-like
Total carotenoids	q-Tot-8-JF	*LOC101509366*	Chr 8 (2,047,214–2,049,321)	Protein stay-green chloroplastic-like
CDC Cory × CDC Jade				
β-carotene	q-Crt-8-CJ	*LOC101509366*	Chr 8 (2,047,214–2,049,321)	Protein stay-green chloroplastic-like
Violaxanthin	q-Vio-8-CJ	*LOC101509366*	Chr 8 (2,047,214–2,049,321)	Protein stay-green chloroplastic-like
Lutein	q-Lut-8-CJ	*LOC101509366*	Chr 8 (2,047,214–2,049,321)	Protein stay-green chloroplastic-like
Total carotenoids	q-Tot-8-CJ	*LOC101509366*	Chr 8 (2,047,214–2,049,321)	Protein stay-green chloroplastic-like

both cotyledon color and β-carotene concentration; this region might be critical for marker assisted selection in chickpea breeding for provitamin A improvement.

The results showed that chickpea seeds with darker green and orange cotyledon color had the highest provitamin A and non-provitamin A carotenoids in all three populations. This finding was consistent with the results obtained by Serrano et al. (2017).

In a cross between ICC 4475, an orange cotyledon chickpea cultivar, and CDC Jade, a green cotyledon cultivar, the progeny had the highest levels of all carotenoids among the three populations (Table 2 and Table 3). As such, chickpea breeders may focus on individuals with darker green or orange cotyledon colors to develop cultivars with elevated provitamin A carotenoids.

The results that cotyledon color is highly associated with provitamin A concentration suggests that the measurement of color density in chickpeas may work for quick detection of provitamin A in the seeds. In case of wheat, two factors, including yellow flour color and yellow pigment content, were used as an alternative to the costly HPLC method for detecting carotenoids (Zhai et al., 2016). Visual selection for carotenoids has been successfully practiced in maize (Burt et al., 2013) and it is reported that a dark orange endosperm is a target trait in breeding for provitamin A biofortification in maize (Owens et al., 2014).

Overall, at least one QTL with a PVE of more than 13% was found for each carotenoid component in each population in the current study. According to the results obtained in this study, QTLs with moderate to large effects control carotenogenesis in chickpea. Similar findings have been reported in maize (Chander et al., 2008; Kandianis et al., 2013).

In conclusion, high-density genetic maps were developed for three chickpea F_2 populations from the crosses CDC Jade × CDC Frontier, CDC Cory × CDC Jade, and ICC4475 × CDC Jade. The QTLs responsible for the concentrations of the carotenoids violaxanthin, lutein, zeaxanthin, β-carotene, and β-cryptoxanthin were identified in this study. Cotyledon color, as a monogenic trait, was mapped on LG8 in all three populations. The *SGR* gene was found within the QTLs for violaxanthin (Vio-8-JF), β-carotene (q-Crt-8-JF), β-cryptoxanthin (q-Cryp-8-JF), and total carotenoids (q-Tot-8-JF) in the CDC Jade × CDC Frontier population and QTLs for violaxanthin (Vio-8-CJ), lutein (Lut-8-CJ), β-carotene (q-Crt-8-CJ), and total carotenoids (q-Tot-8-CJ) in the CDC Cory × CDC Jade population. The majority of the QTLs associated with carotenoids were located on LG8 close to the genomic region associated with cotyledon color in all three F_2 populations. A significant positive relationship between cotyledon color and carotenoid concentration was found. Crossing chickpea cultivars with green and yellow cotyledons was found to be an effective way to map carotenoid components, especially for the provitamin A concentration. Markers associated with QTLs that accounted for high PVE thus have the potential for use to aid selection in chickpea breeding.

Supplemental Information

Supplementary File S1. Means comparison of total carotenoid concentration (μg g^{-1}) ± SE in three F_2 populations of chickpea based on Fisher's least significant difference (LSD) test at the significance level of 5%.

Supplementary File S2. (A) The concentration (μg g^{-1}) of carotenoids including violaxanthin, lutein, zeaxanthin, β-carotene, β-cryptoxanthin, and total carotenoids ± SE measured by HPLC in seed samples of 120 $F_{2:3}$ families derived from a cross between CDC Jade and CDC Frontier. Three different cotyledon classes including Y (yellow), G (green), and S (segregating) are shown for each family. (B) The concentration (μg g^{-1}) of carotenoids including violaxanthin, lutein, zeaxanthin, β-carotene, β-cryptoxanthin, and total carotenoids ± SE measured by HPLC in seed samples of 120 $F_{2:3}$ families derived from a cross between CDC Cory and CDC Jade. Three different cotyledon classes including Y (yellow), G (green), and S (segregating) are shown for each family. (C) The concentration (μg g^{-1}) of carotenoids including violaxanthin, lutein, zeaxanthin, β-carotene, β-cryptoxanthin, and total carotenoids ± SE measured by HPLC in seed samples of 120 $F_{2:3}$ families derived from a cross between ICC 4475 and CDC Jade. Three different

cotyledon classes including Y (yellow), G (green), and S (segregating) are shown for each family.

Supplementary File S3. Frequency distribution of the concentration (μg g^{-1}) of violaxanthin (A), zeaxanthin (B), β-carotene (C), β-cryptoxanthin (D), lutein (E), and total carotenoids (F) measured by HPLC in seed samples of three chickpea $F_{2:3}$ populations: CDC Jade × CDC Frontier (JF), CDC Cory × CDC Jade (CJ), and ICC4475 × CDC Jade (IJ). The concentration of each component in the parents is indicated. No β-cryptoxanthin was detected in seed samples of any parent.

Supplementary File S4. The genetic and physical locations of the SNP markers in each population.

Conflict of Interest Disclosure

The authors declare that there is no conflict of interest.

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