Genetic Analysis of Carotenoid Biosynthesis in Chickpea (*Cicer arietinum* L.) Seeds

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ABSTRACT

Vitamin A deficiency is a worldwide problem especially in the third world countries. Improvement of carotenoid levels in the edible parts of the crops has been one of the major objectives of many plant breeding programs. Chickpea (*Cicer arietinum* L.) is an important source of carotenoids. The availability of diverse germplasm resources along with the availability of its genome sequence, makes chickpea an ideal object for studying carotenogenesis in pulses. The objectives of this research were: 1) to identify the genomic regions associated with carotenoid concentration in diverse chickpea accessions and in populations derived from biparental crosses, 2) to examine the effects of environment on carotenogenesis, 3) to examine the relationship between cotyledon colour and carotenoid concentration, and 4) to examine the expression patterns of the genes involved in carotenoid biosynthesis during seed development. A genotypic panel of 172 chickpea accessions was evaluated in 2015 and 2016 with one location per year in Saskatchewan, Canada. The effects of genotype and environment were significant on the concentration of each carotenoid component. The mean and range for the concentration of each component based on the average of two-years data are as follows: 10.14 and 3.5-28.2 $\mu g\ g^{-1}$ for lutein, 0.37 and 0-3.04 $\mu g\ g^{-1}$ for violaxanthin, 1.65 and 0.27-2.84 $\mu g\ g^{-1}$ for zeaxanthin, 0.09 and 0-2.5 $\mu g\ g^{-1}$ for β-carotene respectively.

The chickpea genotypic panel consisted of two major subpopulations, kabuli and desi groups, along with an admixture group. Genome-wide association analysis revealed that the genes in the primary steps of carotenoid biosynthesis and those involved in apo-carotenoid production had significant associations with carotenoid concentration in chickpea. Three F_2 populations derived from crossing cultivars with green and yellow cotyledons were used to identify QTL associated with carotenoids. Five to eight QTLs responsible for different carotenoid components were identified in each population. In all three populations, the highest phenotypic variation explained by QTL was found for the β-carotene concentration. Cotyledon colour (CotCol) was mapped on linkage group 8 in each population. A positive and significant relationship between cotyledon colour and carotenoid concentration was identified in this experiment. The structure, genomic location, and copy number of 29 genes involved in carotenoid and isoprenoid pathways were retrieved in the chickpea genome. Two missense mutations were found in zeta carotene isomerase (*ZISO2*) in CDC Verano, a green cotyledon kabuli cultivar, which might explain the

higher carotenoid concentration in this cultivar. The expression patterns of 19 genes from the carotenoid pathway were analyzed in five chickpea cultivars at different seed developmental stages. The highest expression level from all the genes was observed at eight and 16 days post-anthesis across the five cultivars. The highest carotenoid concentration and expression levels of the carotenoid genes were found in CDC Jade, a desi cultivar with green cotyledons. Based on gene expression analysis, the desaturation and isomerisation reactions positively affected the carotenoid concentration, while hydroxylation adversely affected the carotenoid concentration. The results from this research could help breeders to develop chickpea cultivars with improved carotenoid/provitamin A levels through molecular breeding.

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LIST OF ABBREVIATIONS

ABA - Abscisic acid

Add - Additive

ANOVA - Analysis of variance

BCH - β-carotene hydroxylase

BCO - β,β-carotene 15,15′-monooxygenase

BHT - Butylated hydroxytoluene

CCD - Carotenoid cleavage dioxygenases

CCS - Capsanthin-capsorubin synthase

CDC - Crop Development Centre

CI - Confidence interval

cM - Centimorgans

CRTISO - Carotenoid isomerase

CT - Cycle threshold

CTAB - Hexadecyltrimethylammonium bromide

CYP97A - Cytochrome P450-type β-hydroxylase

CYP97C - P450-type monooxygenase

DCM - Dichloromethane

DMAPP - Dimethylallyl diphosphate

Dom - Dominance

DPA - Days post-anthesis

DXP - Deoxy-D-xylulose 5-phosphate

DXR - Deoxy-D-xylulose 5-phosphate reductoisomerase

DXS - Deoxy-D-xylulose 5-phosphate synthase

FDR - False discovery rate

GGPP - Geranyl-geranyl diphosphate

GS - Genomic selection

GWAS – Genome-wide association study

H^2- Broad sense heritability

HDR - 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase

HPLC - High-performance liquid chromatography

ICRISAT - International Crops Research Institute for the Semi-Arid Tropics

IPP - Isopentenyl diphosphate

LCYB - Lycopene β-cyclase

LCYE - Lycopene ε-cyclase

LD - Linkage disequilibrium

LG - Linkage group

LOD - Logarithm of odds

LSD - Least significant difference

MAS - Marker-assisted selection

MEP - Methylerythritol 4-phosphate

MLM - Mixed linear model

MVA - Mevalonic acid

NCED - 9-cis-epoxycarotenoid dioxygenase

NJ - Neighbor joining

NMR - Nuclear magnetic resonance

NXS - Neoxanthin synthase

PCA - Principal component analysis

PDS - Phytoene desaturase

PSY - Phytoene synthase

PVE - Phenotypic variance explained

qPCR - Quantitative polymerase chain reaction

QTL - Quantitative trait loci

RCBD - Randomized complete block design

SAS - Statistical analysis software

SE - Standard error

SNP- Single nucleotide polymorphism

UHPSFC - Ultra-high performance supercritical fluid chromatography

VDE - Violaxanthin de-epoxidase

ZDS - ζ-carotene desaturase

ZEP - Zeaxanthin epoxid

CHAPTER 1. INTRODUCTION

Chickpea (*Cicer arietinum* L.*;* 2n= 2x= 16) is a pulse crop with a relatively small genome size (740 Mb; Arumuganathan and Earle, 1991). It is a self-pollinated crop with cleistogamous flowers and life cycle of 3-4 months (Srinivasan and Gaur, 2011). Chickpea is the second most important food legume after common bean in terms of production (Jukanti et al., 2012). Chickpea is considered an essential food and consumed as an alternative to meat in many countries in the semi-arid tropics (FAOSTAT, 2012). The potential of chickpea for nitrogen fixation along with its nutritional value makes it a very important crop in many developing countries (Jukanti et al., 2012). Currently, chickpea is cultivated in 59 countries and its production has increased from 7 million tonnes in 1990 to 13.98 million tonnes in 2016 (FAO, 2016). The major chickpea producing countries include India, Australia, Pakistan, Turkey, Myanmar, Ethiopia, Iran, USA, and Canada, with India being by far the largest producer and consumer. About 99% of chickpea production in Canada was from Saskatchewan in 2014 (Saskatchewan Pulse Growers, 2018).

Chickpea is a good source of vitamins such as riboflavin, niacin, thiamin and β-carotene with provitamin A properties (Cabrera et al., 2003; Abbo et al., 2005). The level of provitamin A in chickpea is higher than in the first 'Golden rice' and red coloured wheat (Kruger and Reed 1988; Ye et al., 2000; USDA, 2010; Fernández-Marín et al., 2014). The antioxidant carotenoids in chickpea may also prevent some cancers (Mathers, 2002). Children with vitamin A deficiency may get xerophthalmia which can cause blindness. Vitamin A deficiency may also put people at risk of diarrheal disease and malaria (ACC/SCN, 2000; World Health Organization, 2009).

One of the important traits in chickpea is cotyledon colour. The cotyledon colour in chickpea has been reported to be associated with carotenoid content (Ashokkumar et al., 2015). Substantial phenotypic variation for cotyledon colours ranging from beige, yellow, light orange to green exists in chickpea, which makes chickpea an ideal model for studying the biosynthesis of seed carotenoids. More than 80% of lutein, zeaxanthin, and β-carotene were present in the cotyledon of chickpea (Ashokkumar et al., 2014). High levels of total lutein, zeaxanthin and β-carotene were associated with darker yellow to green cotyledon colour (Ashokkumar et al., 2015). The colour determining genes and the genes involved in the carotenoid biosynthesis pathway have not been studied in chickpea or other pulses. Segregation analysis in lentil showed that cotyledon colour is a qualitative trait and is controlled by a single or few genes (Slinkard, 1978; Bakhsh et

al., 2013). Abbo et al. (2005) identified a few QTLs associated with provitamin A in chickpea; however, no further analysis was reported. QTL and candidate genes responsible for carotenoid content are well known in other crops including maize (Yan et al., 2010; Chandler et al., 2013; Owens et al., 2014), wheat (Pozniak et al., 2007), sweet potato (Cervantes-Flores et al., 2011), cassava (Welsch et al., 2010) and potato (Wolters et al., 2010; Campbell et al., 2014). The availability of the whole genome assembly of chickpea (Varshney et al., 2013a) will facilitate studying candidate genes responsible for carotenoid biosynthesis in this crop.

The results from the research in this area may allow manipulating of carotenoid pathways in chickpea and open opportunities for chickpea breeders to develop cultivars with improved carotenoid content in seeds as whole food to enhance the nutritional quality for humans and animals.

1.1. Hypotheses

The research was conducted to test the following hypotheses: 1) carotenoid concentration in chickpea seeds is affected by environment and is controlled by a moderate number of genomic loci, 2) pigmentation of chickpea cotyledons is related to carotenoid concentration in seeds, and 3) the genes in the carotenoid pathway which produce the main precursor play a key role in carotenoid production in chickpea seeds.

1.2. Research objectives

1. To identify the genomic regions responsible for carotenoid concentration in diverse chickpea accessions through genome-wide association study.
2. To examine the environmental effects on carotenoid concentration in diverse chickpea accessions.
3. To determine the relationship between cotyledon colour and carotenoid concentration.
4. To identify the QTLs associated with carotenoid content using three chickpea populations.
5. To examine the candidate genes associated with carotenoid biosynthesis and their expression profiles in chickpea seeds at different developmental stages.

The research was divided into three studies. Study I addressed the objectives 1 and 2. A panel of 172 chickpea accessions were grown in replicated trials in Elrose, SK in 2015 and in Limerick, SK in 2016. The seeds were analyzed for the carotenoid concentration using high-performance liquid chromatography (HPLC) technique. Genotyping was done using 50K Axiom® *CicerSNP* Array. Genome-wide association analysis was completed to identify the genomic regions associated with carotenoid concentration in chickpea seeds. Objectives 3 and 4 were addressed in study II. QTL analysis for carotenoid concentration was done using three F_2 populations derived from crossing cultivars with contrasting cotyledon colours. Carotenoid analysis and genotyping were done as described in study I. The relationships between carotenoid concentration and cotyledon colour was examined in study II. The last objective was addressed in study III. The expression of 19 genes that were selected based on the results from the mapping study and the literature, along with sequence analysis among the 32 genes involved in the carotenoid and isoprenoid pathways, were assessed at different seed developmental stages in five chickpea cultivars.

This research is presented in manuscript format in chapters 3, 4 and 5 of this thesis. Characterizing the genetic basis of the carotenoid pathway will provide fundamental information for chickpea breeders to develop cultivars with enhanced carotenoid concentration.

CHAPTER 2. LITERATURE REVIEW

2.1. Chickpea origin and distribution

Chickpea (*Cicer arietinum* L.) is a self-pollinated, diploid (2n= 2x= 16) crop with a relatively small genome size (740 Mb; Arumuganathan and Earle, 1991). Chickpea belongs to the cool-season legumes with good adaptability to semi-arid tropics (Bejiga et al., 2006). There are two main centers of origin for chickpea: Southwest Asia and the Mediterranean as the primary center, and Ethiopia as the secondary center. Turkey and Syria are the most probable origins of this crop (Turrill, 1926; Mangelsdorf, 1952). Based on the Neolithic archeological evidence (Tanno and Willcox, 2006), domestication of chickpea happened in the Fertile Crescent which covers a wide area from south-east Turkey, Iraq, Jordan, Israel to western Iran (Diamond, 1997). Based on the limited geographical distribution and the narrow genetic diversity of ssp. *arietinum*, it was suggested that chickpea domestication happened as a single event (Moreno and Cubero, 1978). Chickpea's wild ancestor (*Cicer arietinum* ssp. *reticulatum*) was found in south-east Turkey (Ladizinsky and Adler, 1976). Molecular analysis revealed *C. reticulatum* as the wild progenitor of cultivated chickpea (Zohary et al., 2012). Molecular marker analyses on the wild and the cultivated chickpeas showed that these two groups do not share common geographical origin suggesting that human disseminated chickpea seeds from its original region (Iruela et al., 2002).

The chickpea growing region was limited to semi-arid areas for many years (Kumar and Abbo, 2001). Global chickpea production increased from 11.7 Mt to 13.7 Mt over a three year period (2011-2014). The largest chickpea producers are India, Australia, Pakistan, Turkey, Myanmar, Ethiopia, Iran, USA, and Canada (FAOSTAT, 2015). After it was introduced to the northern Great Plains, chickpea adapted very well and became an important crop in North America (Gan and Noble, 2000). The expansion of chickpea cultivation in USA, Canada, and Australia is, for the most part, due to its potential for nitrogen fixation and its economic importance compared to cereals (Croser et al., 2003; Warkentin et al., 2003; Cutforth et al., 2007). Chickpea was introduced to the Canadian prairies to fulfill the need for crop rotation and diversification. Saskatchewan is the main chickpea producer in Canada and the area under chickpea cultivation in this province was close to 60,000 ha in 2017 (Specialty Crop Report, 2017). Saskatchewan farmers grow chickpea in rotation with cereals and oilseeds to benefit from water-use efficiency (Miller et

al., 2003), high yield and protein content (Gan et al., 2003), and less greenhouse emission (Lemke et al., 2007).

Many chickpea cultivars have been released by the Crop Development Centre (CDC) of the University of Saskatchewan. The main constraints for chickpea improvement are late maturity and ascochyta blight disease pressure that put its production at risk (Warkentin et al., 2003; Upadhyaya et al., 2015; Sharma and Ghosh, 2016). Anbessa et al. (2007) proposed to incorporate two traits including early flowering and double podding to resolve the late maturity issue and improve productivity. There are many reports about QTLs associated with ascochyta blight resistance in different chickpea populations (Udupa and Baum, 2003; Lichtenzveig et al., 2006; Anbessa et al., 2009; Daba et al., 2016). The introgression of double podding and resistance to ascochyta blight in chickpea was performed by Tar'an et al. (2013) using marker-assisted backcrossing.

2.2. Genetic diversity in chickpea

Chickpea has two common types namely kabuli and desi that are different in seed shape, seed coat colour, and size. The kabuli chickpeas were evolved from the desi type through selection for zero tannin content and white flower in the Mediterranean basin (van der Maesen, 1972; Moreno and Cubero, 1978; Jana and Singh, 1993). Kabuli chickpea, also called garbanzo bean, has larger, cream-coloured seeds and thin seed coat. Kabuli chickpeas are commonly consumed as whole seeds, while the desi type has a smaller, darker coloured seed with a thick seed coat that is usually consumed after dehulling and milling (van der Maesen, 1989; Bejiga and van der Maesen 2006; Cobos et al., 2007). The weight of kabuli seeds is typically higher per 1000 seeds compared to desi types (van der Maessen, 1972). Another difference is pigmentation in stem and flower: the desi type has purple flower petals, whereas kabuli chickpea has white petals. The desi type might come in black and green seed coat and rare blue flower (Pundir et al., 1985). There is also the pea-shaped type of desi that has round seeds like pea and comes in small to medium size (Yadav et al., 2007). The desi type is commonly distributed in South and Southeast Asia and to some extent in Ethiopia, Mexico, and Iran, but kabuli type is mostly grown in the West Asia and the Mediterranean regions (Purushothaman et al., 2014). The market price of kabuli chickpea is usually higher than that of desi type (Agarwal et al., 2012).

An understanding of chickpea genetic diversity will facilitate the crop improvement. Genetic resources like a collection of germplasm can crucially help breeding programs to produce

superior cultivars (Upadhyaya et al., 2008). There are two main CGIAR (Consultative Group on International Agricultural Research) centers for chickpea collection and research: The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) with 17,258 accessions (17,123 cultivated and 135 wild) and the International Center for Agricultural Research in the Dry Areas (ICARDA) with 12,647 accessions (12,343 cultivated and 304 wild; Kumar et al., 2004; Upadhyaya et al., 2008). Despite considerable number of accessions available for chickpea, their utilization has been limited. To increase the use of genetic diversity available in the gene banks, core and mini-core collections were developed in various crops as well as in chickpea (Brown, 1989; Upadhyaya et al., 2001). A total of 3,000 chickpea accessions were assembled and used for developing a composite collection. The collection included accessions from ICRISAT (65.2%), ICARDA (23.6%), breeding lines (1.3%), trait-specific chickpeas (8%) and wild species (0.66%; Upadhyaya et al., 2001; Dwivedi et al., 2005; Upadhyaya et al., 2006). Interspecific hybridization is one of the strategies that exploits the genetic variation of wild relatives with favourable traits to improve the genetic basis in cultivated chickpea (Saxena et al., 2014a).

Genetic diversity and kinship studies including agronomic traits, karyotyping, seed storage protein and isozyme profiling have been done for different *Cicer* species including the annual and perennial chickpeas (Dwivedi et al., 2005; Singh et al., 2008). Different DNA based markers such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), inter simple sequence repeat (ISSR), and simple sequence repeat (SSR) were used for genetic diversity study in chickpea. The results confirmed that the wild chickpeas had higher diversity than the cultivated chickpeas (Sharma et al., 1995; Sant et al., 1999; Iruela et al., 2002; Sudupak et al., 2002; Rajesh et al., 2003; Shan et al., 2005; Varshney et al., 2014). The advance in next generation sequencing and the availability of chickpea genome sequences (Jain et al., 2013;Varshney et al., 2013a) allowed the identification and extensive use of single nucleotide polymorphism (SNP) markers for assessing genetic diversity in chickpea (Kujur et al., 2013; Roorkiwal et al., 2014; Bajaj et al., 2015). More recently, genotyping by sequencing (GBS) was used for genetic variation analysis in a chickpea collection including Apulian black chickpea (Pavan et al., 2017). One approach to increase genetic diversity is collecting and protecting crop wild relatives in plants. A total of 1,210 accessions, a combination of wild and cultivated chickpeas collected from Turkey, Canada, Ethiopia and India were preserved and used to maximize genetic diversity in chickpea (Von Wettberg et al., 2018).

2.3. Genomic resource development in chickpea

The genomic assisted breeding approach allows scientists to use markers to improve chickpea cultivars for traits such as yield (Varshney et al., 2013c), resistance to fusarium and ascochyta (Varshney et al., 2014) and yield-related traits (Roorkiwal et al., 2016). Several approaches have been implemented in developing molecular marker resources in chickpea.

Using ESTs (expressed sequence tags) libraries resulted in the identification of more than 3,000 SSR markers (Varshney, 2016). Arrays for chickpea genotyping using diversity arrays technology (DArT) were developed by collaborative work between ICRICAT and DArT Pty Ltd, Australia (Varshney et al., 2010). A total of 2,005 SNPs were developed that were optimized for Kompetitive Allele Specific PCR (KASP) in chickpea (Hiremath et al., 2012). Golden-Gate and VeraCode assays were developed and successfully used for SNP genotyping in chickpea (Roorkiwal et al., 2013; Diapari et al., 2014).

The whole genome sequence of CDC Frontier chickpea, the Canadian kabuli was reported by Varshney et al. (2013a). The draft (~ 738-Mb) contained an estimated 28,269 genes. Moreover, 90 chickpeas including cultivars and wild accessions (Varshney et al., 2016), 35 cultivars as parents in mapping population, 300 references, and 100 elite chickpea lines have been sequenced. ICRICAT sequencing project for 3,000 chickpeas (The 3,000 Chickpea Genome Sequencing Initiative) started in 2014 to identify the important alleles for chickpea breeding (Varshney, 2016). The sequence of the chickpea genome is available in the International Chickpea Genome Sequence Consortium (ICGSC, www.icrisat.org/gt-bt/ICGGC/Homepage.htm), which is coordinated by ICRICAT (Varshney, 2016). In addition, the draft genome assembly of ICC4958, a drought-tolerant desi germplasm, was developed based on next generation sequencing data. It is predicted that 954 legume-specific genes are present in this assembly (Jain et al., 2013). Based on the chromosomal genomics approach, the physical genomes of kabuli and desi chickpeas are quite similar. The previously reported differences were mainly due to errors in the desi genome assembly, such as the misplacement of whole chromosomes, portions of chromosomes and the inclusion of a large portion of sequence assembly which were not related to chickpea genome (Ruperao et al., 2014). The availability of whole genome sequences will provide the opportunity to use markers like SNPs for high-throughput genotyping of mapping populations or germplasm collections (Varshney, 2010; Hiremath et al., 2012). Genome scanning for SNPs identification, RNA-seq analysis, and characterization of other polymorphisms like INDELs are useful for

genotyping resource development in chickpea (Varshney et al., 2013a). Candidate gene analysis and whole genome scanning were successfully deployed in an association study for identifying markers related to drought tolerance in chickpea (Thudi et al., 2014).

The hybrid transcriptome assembly was successfully used for advance genetic research in chickpea to identify the elite varieties (Kudapa et al., 2014). In addition, a high-density genetic map has been developed in chickpea using RAD-Seq GBS and Illumina GoldenGate (Deokar et al., 2014). The alignment of the map with the kabuli genome sequence showed an overall conserved marker order with some localized inversions. The alignment also allowed the estimation of genome-wide recombination rates and hot spot regions in the chickpea genome (Deokar et al., 2014). Other SNP-based linkage map in chickpea had also been developed (Gaur et al., 2015), which can be anchored to the genome sequences of the kabuli or desi chickpeas. Recently, a high-density Axiom® *CicerSNP* chip with more than 50K SNPs has been designed and validated for chickpea mapping (Roorkiwal et al., 2017).

2.4. Nutritional and anti-nutritional composition of chickpea seeds

Chickpea seed has the highest concentration of carbohydrates among pulses. The major carbohydrate in chickpea is starch (30-57%), which is divided into two types of glucose polymers including linear amylose and branched amylopectin (Saini and Knights, 1984; Salgado et al., 2001). The desi and kabuli seeds consisted of 20–41% and 23–47% amylose, respectively, while the remaining starch comprised of amylopectin (Saini and Knights, 1984; Hoover and Ratnayake, 2002). The other important carbohydrates in chickpea seeds included ribose, glucose, sucrose, maltose, stachyose, and raffinose (Han and Baik, 2006). Although chickpea is a good source of protein and energy, there are some anti-nutritional factors (ANFs) present including phytic acid (PA), saponin, raffinose family oligosaccharides (RFOs) and enzyme inhibitors (Roy et al., 2010). Phytic acid can bind to micronutrients such as iron, zinc, calcium, and magnesium and reduce their bioavailabity in the human diet (Fox and Eberl, 2002). The concentration of PA in kabuli is higher than in desi genotypes (Bueckert et al., 2011). The side effect of consuming RFO in high concentration is stomach discomfort, because its fermentation produces CO_2 and, in lesser quantity, methane gases. However, consumption of ROF in low concentration positively affects the growth of beneficial intestinal microflora (Martínez-Villaluenga et al., 2008).

The other ANFs in chickpea are saponin, inhibitors of trypsin, chymotrypsin, and α-amylase (Muzquiz and Wood 2007; Roy et al., 2010). Chickpea is also known as a good source of

vitamins including riboflavin, niacin, thiamin, and β-carotene as the precursor of vitamin A (Cabrera et al., 2003; Abbo et al., 2005; Ashokkumar et al., 2015).

2.5. Protein in chickpea

Chickpea seeds consist mainly of carbohydrates and proteins and are good source of vitamins and minerals (Wood and Grusak, 2007; Chibbar et al., 2010). Seed protein content is among the factors that determine seed quality in chickpea (Upadhyaya et al., 2016a). Chickpea seeds can also be used as protein-rich animal feed, while the vegetative biomass can be used as fodder (Saraf et al., 1998). The concentration of starch in both desi and kabuli chickpeas has a negative correlation with seed protein concentration (Frimpong et al., 2009). Chickpea has the best type of protein among legumes with high *in vitro* protein digestibility (IVPD; Jukanti et al., 2012). The average of chickpea total protein content as a percentage of dry seed is 17-22% before and 25.3-28.9% after dehulling, respectively (Maheri-Sis et al., 2010; Jukanti et al., 2012). The storage proteins in chickpea seed consists of a combination of albumin, globulin, and glutelin (Chang et al., 2011). The gene encoding lectin protein in chickpea (CpSL) shared high sequence homology with other lectin genes in legumes (Qureshi et al., 2007). The composition of amino acids in chickpea seed makes it a valuable source of essential amino acids among legumes (Gupta and Kapoor, 1980). The amino acid content such as lysine, phenylalanine, leucine, and valine are found in notable quantities in chickpea. However, sulphur-containing amino acids (methionine and cystine) are limited (Iqbal et al., 2006). Both kabuli and desi types showed almost similar amino acid profile (Wang and Daun, 2004; Wang et al., 2010). There are chickpea landraces that have desirable protein properties that can be used as sources for developing chickpea cultivars with higher protein values (Jauhar, 2006; Natarajan et al., 2012).

The accumulation of storage proteins in chickpea seeds is controlled by different QTLs (Jadhav et al., 2015). An approach for dissection of QTLs for seed protein content was by integrating QTL mapping and GWAS as reported in soybean (Sonah et al., 2015). Genome-wide association study based on simple sequence repeat (SSR) markers in chickpea germplasm showed that four QTLs were associated with protein content and candidate genes like malate synthase and 6-phosphogluconate dehydrogenase were found as the major genes affecting protein content in this crop (Jadhav et al., 2015). Genomic regions associated with seed storage proteins in cultivated chickpeas and landraces were also mapped using proteomic approach (Singh et al., 2016). One of the important genes that affects the seed size in legumes is *BIG SEEDS1 (BS1)*. Down regulation

of *BS1* positively increased the seed size and amino acid content in soybean (Ge et al., 2016). Gene silencing for improvement of seed protein quality had also been used in soybean (Schmidt et al., 2011).

2.6. Fat properties in chickpea

Fat content in chickpea is relatively low (3.8 to 10%), as such chickpea is not considered as an oilseed crop (Gül et al., 2008). The total fat content in chickpeas varies from 2.9 to 7.4% in desi, and 3.4 to 8.8% in kabuli types (Wood and Grusak, 2007). Fat content in chickpea is higher than in lentil, pigeon pea, red kidney and mung bean, as well as cereals like rice and wheat (USDA, 2010). Fatty acids in chickpeas consist of polyunsaturated fatty acids (~ 66%), monounsaturated fatty acids (~ 19%) and saturated fatty acids (~ 15%). Linoleic acid (LA) ranks as the largest fatty acid in chickpea, followed by oleic acid (OA) and palmitic acid. Kabuli chickpea is rich in OA, while desi chickpea is rich in LA (Wang and Daun, 2004; Jukanti et al., 2012). Based on Wijs method, iodine values in chickpea oil are higher than groundnut and *Phaseolus vulgaris,* also its relative index values are higher than in groundnut and soybean (Mabaleha and Yeboah, 2004; Zia-Ul-Haq et al., 2007). The unsaturated fat in chickpea makes it a valuable source of nutrition for people with heart and circulatory problems (Shah et al., 2013). Polyunsaturated fatty acids in chickpea can decrease the concentration of low-density lipoprotein (LDL) cholesterol and total cholesterol in patients with mild hypercholesterolemic (Pittaway et al., 2006).

Oil content is a quantitative trait that was highly affected by genotype and environment and its concentration is negatively correlated with seed size (Dwivedi et al., 1990, 1993). In soybean, 12 major QTLs were identified for oil content (Zhaoming et al., 2017). A high number of genes are involved in oil biosynthesis; for instance, about 850 and 1,582 genes were found in peanut and soybean, respectively (Wang et al., 2017). Biosynthesis of fatty acids occurs in seed plastid using sucrose which is ultimately converted into pyruvate through glycolysis (Hajduch et al., 2011). Next, enzyme pyruvate dehydrogenase catalyzes pyruvate to CO2 and acetyl-CoA in which the latter is converted into malonyl-CoA under the control of acetyl-CoA carboxylase (ACCase). Following this, enzyme fatty acid (FA) synthase produces 16:0 ACP, 18:0 ACP and 18:1 ACP from malonyl-CoA (Chapman and Ohlrogge, 2012; Song et al., 2017). The RNAi technique was used to repress ADP-glucose pyrophosphorylase (*AGP*) in pea, which consequently resulted in the elevation of oil content and a decrease of the starch content in kernels (Weigelt et al., 2009). Transferring the diacylglycerol acyltransferase (*DGAT2A*) gene from the soil fungus

Umbelopsis ramanniana to soybean resulted in an increase in total oil content in mature seeds (Lardizabal et al., 2008). Decreasing the expression level of fatty acid desaturase 2, an important gene in the oil pathway involved in the conversion of oleic acid to linoleic acid in soybean, using RNAi (Wagner et al., 2011) and mutation (Demorest et al., 2016), successfully increased the amount of oleic acid. Mutations using X-ray or EMS were found to be practical methods for improving seed oil content in soybean (Dierking and Bilyeu, 2009; Pham et al., 2010, 2011).

2.7. Zinc and iron in chickpea

The most important minerals in chickpea seeds are iron (Fe), zinc (Zn), magnesium (Mg), and calcium (Ca; FAO, 2002). In addition, chickpea is a reliable source of selenium (Ray et al., 2014). Micronutrients such as Fe and Zn are critical for normal metabolism in humans. Deficiency of micronutrients has been reported in three billion people including children in developing countries (Welch, 2002; Thavarajah and Thavarajah, 2012). These two elements are co-factors in various proteins such as cytochrome, transcription factors, and haemoglobin that are important in growth and development (Welch and Graham, 1999; Blair et al., 2010). Fe and Zn shortage may cause anemia, hypogonadism, dwarfism, orifical and acral dermatitis that are threatening half of the world population (Brown et al., 2002; Welch, 2002). Pulses contain greater concentration of Zn than cereals (Hemalatha et al., 2007). The average amounts of Fe and Zn per 100 g edible protein in chickpea are 3.0–14.3 and 2.2–20 mg, respectively (Wood and Grusak 2006; Ray et al., 2014). Kabuli chickpea has higher Zn, similar Fe, but lower Mg and Ca concentrations compared to desi chickpea (Bueckert et al., 2011). Diapari et al. (2014) reported that the effects of environment on concentration of iron and zinc were significant in chickpea. In rice, approximately 60% of zinc and iron concentration variation was caused by environment (Norton et al., 2014). Environment also significantly affected mineral micronutrient concentration in field pea, chickpea, common bean, and lentil grown in Saskatchewan, Canada (Ray et al., 2014).

Seed embryo development is dependent on Fe (Stacey et al., 2008). In pea, the Fe, concentration decreased from epidermis to inner layers of the seeds (Grillet et al., 2014). The genetic basis of Fe and Zn accumulation is controlled by a complex cellular system. Fe and Zn concentrations in chickpea seeds are quantitative traits governed by QTLs (Diapari et al., 2014; Upadhyaya et al., 2016a). The concentration of these minerals in chickpea seed is highly associated with genes encoding vacuolar protein sorting, late embryogenesis abundant, and yellow stripe-like 1 protein (Upadhyaya et al., 2016a). Genetic studies using recombinant inbred lines of Andean

common beans (*Phaseolus vulgaris* L.) revealed that nine QTLs were responsible for zinc and iron concentrations (Blair et al., 2011). Association mapping using SNPs in lentil (*Lens culinaris* Medik) identified significant markers associated with zinc and iron concentration in the seeds (Khazaei et al., 2017). Phenotyping mineral profiles in seeds can be done using high-throughput ionomics technology which is much more cost-effective than the conventional method (Baxter, 2010).

Gene *OsYSL2* synthesize Fe transporter was reported as one of the important candidate genes involved in iron transportation during seed development (Koike et al., 2004). Suppression of *AtOPT3* negatively affected the Fe concentration in soybean seeds (Stacey et al., 2008). The tissues which are not linked symplastically usually acquired the Fe nutrient through transcription of citrate efflux transporter FRD3 in the embryo protodermis and aleurone layer (Roschzttardtz et al., 2011). The storage form of Fe in legumes is plastidic ferritin (Davila-hicks et al., 2004). Soybean ferritin was used to develop transgenic rice for Fe biofortification purpose (Goto et al., 1999). Another rice cultivar was developed by transformation of nicotianamine synthase 1 (*AtNAS1*), ferritin (*PvFERRITIN*), carotene desaturase (*CRTI*) and phytoene synthase (*ZmPSY*) to improve β-carotene, iron, and zinc levels (Singh et al., 2017).

2.8. Carotenoids

The C40 carotenoids are isoprenoid compounds with more than 700 types in nature (Britton et al., 2004). Most photosynthetic organisms synthesize carotenoids which have various roles such as colour pigments, antioxidant and photoprotective functions (Howitt and Pogson, 2006; Khoo et al., 2011). Carotenoids are categorized into two classes: 1) oxygenated such as lutein, violaxanthin, and neoxanthin and 2) non-oxygenated like β-carotene and lycopene (Dellapenna and Pogson, 2006). β-carotene, lutein, zeaxanthin, β-cryptoxanthin, lycopene, and α-carotene are the most important carotenoids in chickpeas (Figure 2.1 B). These components are a rich source of provitamin A. Among which, β-carotene can be converted into vitamin A more efficiently (Abbo et al., 2005). The key step in vitamin A production is the conversion of β-carotene into two molecules of all-trans-retinal by β,β-carotene 15,15′-monooxygenase (BCO) in the human body (Chichili et al., 2005).

Various species and organ types in plants have different carotenoids. For instance, the majority of provitamin A with one or two β-rings are found in green tissues (Goodwin and Britton, 1988), but non-green tissues have more distinctive composition depending on the plant species

(Tanaka et al., 2008). Important carotenoids in plant leaves and stems include violaxanthin, zeaxanthin, lutein, and antherxanthin (Fraser et al., 1994; Tanaka et al., 2008). The presence of carotenoids in carrot (Baranska et al., 2006a) and sweet potato (Hagenimana et al., 1998) is exceptional as plants normally do not reserve carotenoids in the root.

Ketocarotenoids are a kind of carotenoid whose biosynthesis has been well characterized in marine bacteria (Misawa et al., 1995). Their biosynthesis proceeds by hydroxylation of β-carotene to zeaxanthin, followed by ketolation (Scaife et al., 2012). Among ketocarotenoids, astaxanthin (3,3′-dihydroxy-4, 4′-diketo-β-carotene) has significant commercial value due to its superior antioxidant activity. This component is used for animal feed, natural colourant and cosmetic, and it also inhibits oxidation of low-density lipoprotein in human (Iwamoto et al., 2000). Higher plants, except for Adonis, are unable to biosynthesize ketocarotenoids, because of the absence of β-carotene ketolase (4,4′-oxygenase) gene (Cunningham and Gantt, 2005; 2011). To synthesize astaxanthin in plants, the β-carotene ketolase gene from other organisms including alga and marine bacterium was introduced to different crops (Hasunuma et al., 2008; Jayaraj et al., 2008). There are more than 700 natural carotenoids (Delgado-Vargas et al., 2000; Britton et al. 2004), but only six of them including, α-carotene, β-carotene, lutein, lycopene, zeaxanthin, and astaxanthin are proven to be health-beneficial components (Johnson, 2002). Vitamin A is a substantial element for human body. Over 125 million children and 7 million pregnant women in developing countries suffered vitamin A deficiency (Iannotti et al., 2013). The deficiency can cause severe problems like blindness, childhood malaria, diarrhea, and measles (West and Darnton-Hill, 2008). Consuming animal-derived food and provitamin A carotenoids are two major ways for the human to absorb vitamin A (retinol). Provitamin A carotenoids, those containing β-ring like β-carotene, are present in various crops (von Lintig, 2012a,b; Giuliano, 2017). The β-carotene can reduce the side effects of nutritional deficiency such as night blindness (Haskell et al., 2005). It may also have positive effect on the digestive and immune systems in human (Chew, 1993). There is no report about the role of β-carotene in cancer prevention (Druesne-Pecollo et al., 2010). Consuming food with high zeaxanthin and lutein levels decreased the risk of age-related macular degeneration (Ma et al., 2012). Lycopene is one of the carotenoids whose role in cancer prevention is not clear (Mein and Wang, 2008; Zu et al., 2014).

2.9. Carotenoid biosynthesis pathways

The C_{40} tetraterpenoids carotenoids are present in all photosynthetic tissues (Dellapenna and Pogson, 2006; Grotewold, 2006). Two isoprene isomers including isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are the main components for biosynthesis of different carotenoids as well as plastoquinone chlorophylls, phylloquinone, monoterpenes, gibberellins (GA), and tocopherols (Rodriguez-Concepcion, 2010). Biosynthesis of IPP occurs through two pathways: plastidic methylerythritol 4-phosphate (MEP) and cytosolic mevalonic acid pathway (MVA). MEP pathway is involved in the production of IPP and DMAPP for carotenoid formation in plants (Eisenreich et al., 2001, 2004; Rodriguez-Concepcion and Boronat, 2002). The initial reaction of the MEP pathway produces deoxy-D-xylulose 5-phosphate (DXP) from glyceraldehyde 3-phosphate and pyruvate, which is performed by DXP synthase (*DXS*). Then, DXP reductoisomerase (*DXR*) catalyzes the reduction and rearrangement of DXP for MEP synthesis (Julliard and Douce, 1991). Both enzymes are under the control of a single gene in *Arabidopsis* and its overexpression increases carotenoid content (Estévez et al., 2001; Carretero-Paulet et al., 2006). Next, enzyme 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase (*HDR*) catalyzes the production of two molecules: IPP and DMAPP which are used for yielding the main precursor of isoprenoid molecules, geranyl-geranyl diphosphate (GGPP; Kleing 1989; Lichtenthaler, 1999; Giuliano, 2017). Formation of phytoene as a 15-cis isomer is the initiation of carotenoid biosynthesis which is performed through condensation of two GGPPs by phytoene synthase (*PSY*) which is the rate limiting enzyme in carotenoid biosynthesis (Rodriguez-Concepcion, 2010; Figure 2.1 A). The activity of *PSY* is higher in chloroplast thylakoid membrane over de-etiolation stage (Rodriguez-Villalon et al., 2009; Welsch et al., 2000). Higher activity of *DXS* could elevate *PSY* expression, whereas obstructing MEP pathway leads to lower expression of *PSY* (Rodriguez-Concepcion et al., 2002; Laule et al., 2003). In the next step, phytoene undergoes dehydrogenation reactions catalyzed by two enzymes: phytoene desaturase (*PDS*) and ζ-carotene desaturase (*ZDS*) which produces tetra-cis-lycopene from 15-cis phytoene (Bartley et al., 1991; Albrecht et al., 1995; Chen et al., 2010; Yu et al., 2011). The specific carotenoid isomerase (*CRTISO*) converts tetra-cis lycopene to all-trans-lycopene. This enzyme does need flavin adenine dinucleotide (FAD) for isomerizing cis bonds at 7, 9 and 7′, 9′ positions (Park et al., 2002; Isaacson et al., 2004). Two *CRTISOs* were found in *Arabidopsis*, tomato, and grape which are encoded by single copy gene (Fantini et al., 2013).

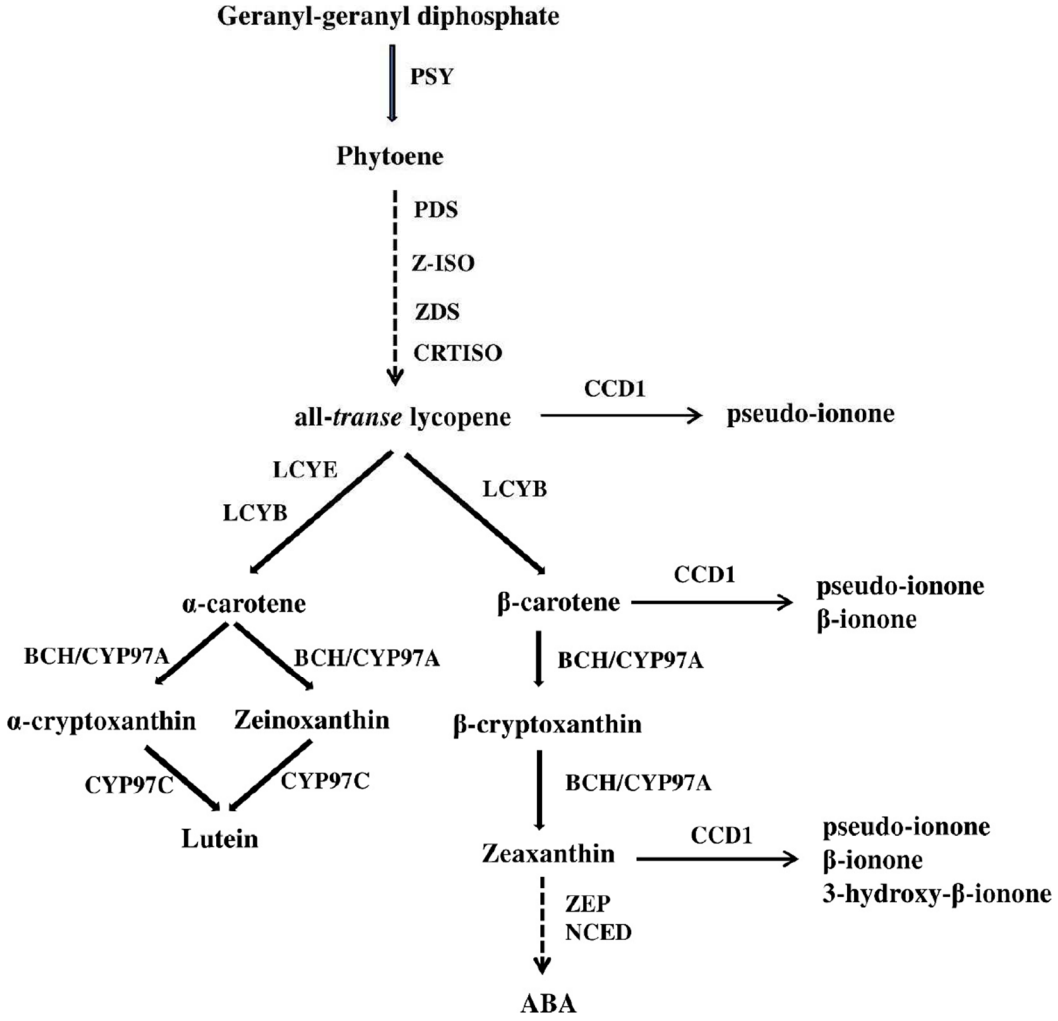
Cyclization of all-trans-lycopene is a substantial step for carotenoid biosynthesis, which increases the diversity of these components. Two enzymes including lycopene β-cyclase (*LCYB*) and lycopene ε-cyclase (*LCYE*) control the cyclization by adding beta and/or epsilon rings to produce β-carotene and α-carotene form lycopene (Cunningham et al., 1993, 1996; Pecker et al., 1996; Ronen et al., 1999). These two enzymes play an important role in the determination of composition and concentration of carotenoids in various crops (Nisar et al., 2015). In pepper family, another type of cyclase activity has been observed which is catalyzed by capsanthin-capsorubin synthase (*CCS*). This enzyme adds a cyclopentane ring to antheraxanthin and violaxanthin which finally converts them to capsanthin and capsorubin respectively (Bouvier et al., 1994; Lefebvre et al., 1998; Gomez-Garcia and Ochoa-Alejo, 2013).

Synthesis of xanthophylls such as lutein and zeaxanthin occurred by hydroxylation of α-carotene and β-carotene. Conversion of β-carotene to zeaxanthin via β-cryptoxanthin is controlled by β-OHase (β-hydroxylase), and two enzymes ε- and β-OHases are responsible for hydroxylation of α-carotene. There are more hydroxylase genes, which have a role in xanthophyll formation in Arabidopsis and tomato. For example, cytochrome P450-type monooxygenase (*CYP97C*) catalyzes the hydroxylation of ε-ring in α-carotene and two enzymes, namely cytochrome P450-type β-hydroxylase (*CYP97A*) and β-carotene hydroxylase (BCH), are responsible for hydroxylation of β-rings in α and β-carotene (Pogson et al., 1996; Sun et al., 1996; Tian and DellaPenna, 2001; Messias et al., 2014). *CYP97A* and *CYP97C* creates protein-protein interaction which makes multienzyme complex for conversion of α-carotene to lutein (Quinlan et al., 2012). Hydroxylation of β-rings in zeaxanthin by zeaxanthin epoxidase (*ZEP*) results in the production of antheraxanthin and then violaxanthin (Chen et al., 2014b; Misra et al., 2006; Figures 2.1 A, B).

Under light stress period, another enzyme violaxanthin de-epoxidase (*VDE*) reverses the reaction and converts violaxanthin to zeaxanthin through a process called xanthophyll cycle. Both *ZEP* and *VDE* are responsible for transportation of small hydrophobic molecules (Hieber et al., 2000; Jahns and Holzwarth, 2012; Latowski et al., 2011). The high light in the lumen of chloroplast leads to lower pH which activates *VDE* enzyme (Pfündel and Bilger, 1994). Then, neoxanthin synthase (*NXS*) converts violaxanthin to neoxanthin, the last carotenoid with β,β branch, in the pathway (Welsch et al., 2008). The 9-*cis*-epoxycarotenoid dioxygenase (*NCED*) cleaves both 9-*cis*-neoxanthin and 9-*cis*-violaxanthin and these cleavage products undergo some modification are finally converted to ABA (Schwartz et al., 1997; Seo and Koshiba, 2002). Carotenoid cleavage

dioxygenases (*CCD*) enzymes produce apocarotenoids from C40 carotenoids which results in a steady-state level of carotenoids (Hannoufa and Hossain, 2012; Li and Yuan, 2013; Figure 2.1 A).

A



B

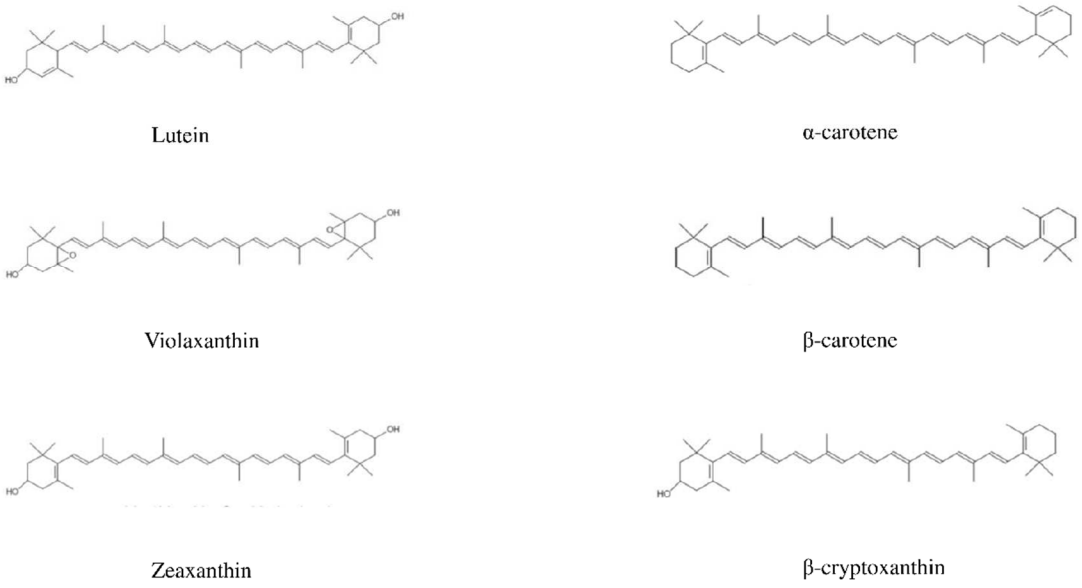


Figure 2.1. A: Carotenoid biosynthetic pathway with precursors and genes including *PSY*, phytoene synthase; *PDS*, phytoene desaturase; *ZDS*, ζ-carotene desaturase; *CRTISO*, carotene isomerase; *Z-ISO*, 15-cis-ζ-carotene isomerase; *LCYB*, lycopene beta cyclase; *LCYE*, lycopene epsilon cyclase; *CYP97A*, cytochrome P450-type β-hydroxylase; *BCH*, β-carotene hydroxylase; *CYP97C*, cytochrome P450-type monooxygenase; *ZEP*, zeaxanthin epoxidase; *NCED*, 9-cis-epoxycarotenoid dioxygenase; ABA, abscisic acid; *CCD1*, carotenoid cleavage dioxygenase 1. Those steps that not shown in this pathway are indicated as dotted lines (adapted from Messias et al., 2014). B: The molecular structure of important carotenoids in plant including α-carotene β-carotene, β-cryptoxanthin, lutein, violaxanthin and zeaxanthin (modified from Meléndez-Martínez et al., 2010).

2.10. Carotenoid accumulation in seeds

Most studies on seed carotegenesis were conducted in maize because the *viviparous* mutants were available in maize (Yan et al., 2010). There is little research on other grain cereals in comparison. The reason might be the naturally very low concentration of carotenoids presents in the endosperm. The stability and accumulation of carotenoids are dependent on the type and size of plastids (Hannoufa and Hossain, 2012; Li and Yuan, 2013). High level of carotenoids was observed in amyloplasts of maize (Howitt and Pogson, 2006). The major carotenoid in wheat (Hentschel et al., 2002), as well as many oilseed crops such as sunflower (McGraw et al., 2001), pumpkin (Matus et al., 1993), and canola (Shewmaker et al., 1999) are lutein. Wild-type maize kernels contain mainly lutein, zeaxanthin, and a small amount of β-carotene (Janick-Buckner et

al., 1999). White and red millet have high level of lutein and zeaxanthin, respectively (McGraw et al., 2001).

Lutein is the most prevalent carotenoid in chickpea and the large proportion was observed in cotyledon of chickpea seeds (Abbo et al., 2005, 2010; Ashokkumar et al., 2014; Figure 2.1 B). The total carotenoid concentration in dry seeds of chickpea (9.08 µg g^{-1}, Abbo et al., 2005) was higher than the genetically-engineered "Golden Rice" endosperm (1.6 µg g^{-1}, Ye et al., 2000) or red coloured wheat (1.8-5.8 µg g^{-1}; Kruger and Reed 1988; USDA, 2010). However, in Golden Rice2, the carotenoid content was increased up to 23-fold (37 µg $g^{-1}$of dry weight) compared to the original product (Paine et al., 2005). Carotenoids have a substantial role in the seed as an antioxidant, limiting seed aging as well as ABA biosynthesis (Maluf et al., 1997; Pinzino et al., 1999; Calucci et al., 2004). The antioxidant reactions are noticeable over seed germination which consequently prevent peroxidase activities (Rogozhin et al., 2001). Moreover, a negative correlation between lutein content and reactive oxygen species (ROS) level was observed in the seed (Galleschi et al., 2002).

2.11. Carotenoids and photosynthesis

Thylakoid membranes and plastoglobuli in chloroplasts are the main places for storing carotenoids in green tissues (Nisar et al., 2015). Carotenoids stabilize the chlorophyll membranes and are involved in light harvesting and photoprotection. They absorb lights in the range of 450-570 nm and send it to chlorophyll (Demmig-Adams et al., 1996). Protection of photosynthetic organs by carotenoids are performed in several ways such as quenching single and triple oxygen and dissipation of extra light which takes by antenna (Demmig-Adams et al., 1996; Kim and DellaPenna, 2006). Upon the oxidation period by ROS, carotenoids work as oxidative stress ''sensor'' and ''signals'' (Havaux, 2014; Ramel et al., 2012ab; Shumbe et al., 2014).

Important components in photosynthesis such as carotenoids, tocopherols, plastoquinone, and the phytol moiety of chlorophylls are synthesized from GGPP as the common precursors. There are many hormones and signaling molecules that are produced from different carotenoids. For example, cytokinins, gibberellins, strigolactones, and ABA are synthesized from DMAPP, GGPP, β-carotene, and 9-cis-epoxyxanthophylls, respectively (Giuliano, 2017). The important role of carotenoids in photosynthesis should receive special attention from scientists interested in metabolic engineering because undesired phenotype or perturbation of metabolism was observed

in various cases. For instance, in tomato, overexpression of *35S:PSY* caused the reduction in gibberellin biosynthesis that consequently resulted in dwarf tomato (Fray et al., 1995).

2.12. Provitamin A biofortification in various crops

To optimize carotenoid accumulation through genetic engineering, the biosynthesis and sequestration of carotenoids should be considered simultaneously (Nogueira et al., 2013). The increased level of carotenoid products is normally associated with both the chloroplastidic and the cytosolic pathways including mevalonate, carotenoids, sterols, and squalene, as well as triacyl-glycerides (Kumar et al., 2012). There are four strategies for carotenoids biofortification including push, block, metabolic sink increasing, and post-harvest stability in different crops (Giuliano, et al., 2017).

2.12.1. Push strategy

Overexpression of enzymes in the carotenoid pathway is a push strategy that was applied for β-carotene biofortification in some crops such as golden canola (Shewmaker et al., 1999), rice (Paine et al., 2005; Ye et al., 2000), potatoes (Diretto et al., 2007a), maize (Zhu et al., 2008b), cassava (Welsch et al., 2010), wheat (Wang et al., 2014a), sorghum (Che et al., 2016), and banana (Paul et al., 2017). The transformation of three genes including phytoene synthase (*CrtB*), phytoene desaturase (*CrtI*) and lycopene β-cyclase (*CrtY*) from *Erwinia*, under tubers specific or constitutive promoter control, was applied in potato. The total carotenoid concentration increased up to 20-fold in the transgenic potato (Diretto et al., 2007b). The transformation of cyanobacterial *crtO* ketolase to tobacco for ketocarotenoid biosynthesis was performed by Zhu et al. (2007). In addition, introducing two genes encoding *CrtW* (β-carotene ketolase) and *CrtZ* (β-carotene hydroxylase) from marine bacterium *Brevundimonas* sp. to tobacco resulted in higher astaxanthin production compared to previous studies (Hasunuma et al., 2008). Ketocarotenoid biosynthetic pathway in carrot through transformation of β-carotene ketolase isolated from the alga *Haematococcus pluvialis,* showed that carrot is an ideal case for biopharming ketocarotenoid production (Jayaraj et al., 2008). Transgenic maize with 169-fold the normal amount of β-carotene in kernels suggested that conventional breeding is not the only method for provitamin A improvement in cereals (Naqvi et al., 2009). Development of soybean (*Glycine max* L. Merr. cv. Kwangan) with higher β-carotene was done through the seed-specific over-expression of two carotenoid biosynthetic genes, namely *Capsicum* phytoene synthase and *Pantoea* carotene desaturase (Kim et al., 2012).

Co-expression of β-carotene ketolase from *Chlamydomonas reinhardtii* and β-carotene hydroxylase from *Haematococcus pluvialis*, increased the level of astaxanthin up to 3.12 mg/g in tomato leaves (Huang et al., 2013). Multiple transformations of bacterial carotenoid genes like isopentenyl pyrophosphate isomerase (*IPI*) and β-carotene ketolase (*CrtW*) to canola and *Lilium* were performed through a single *Agrobacterium* construct (Azadi et al., 2010; Fujisawa et al., 2009). In addition, internal ribosomal entry site (IRES) from a plant virus, and self-cleaving protein sequence (2A) from an animal virus have been used for expression of down-stream genes in multiple gene transformation approaches to elevate β-carotene levels in plants (Ha et al., 2010; Kim et al., 2012). Transformation of whole cytoplasmic mevalonate pathway for biosynthesis of IPP in tobacco (Kumar et al., 2012) was considered as an innovative strategy for carotenoid improvement in plants. However, overexpression of the genes in the carotenoid pathway may decrease the precursor level, consequently limits the synthesis of the desired product (Giuliano, 2017).

2.12.2. Blocking

The second approach for carotenoid biofortification is blocking that can be achieved through gene silencing methods. In potato, silencing the β-carotene hydroxylase and lycopene epsilon cyclase resulted in higher carotenoid and β-carotene content in tubers (Diretto et al., 2006, 2007c). RNA interference (RNAi) of carotenoid cleavage dioxygenase (*CCD4*) increased the accumulation of carotenoid content, two to five fold higher than that of the control plants, suggesting that *CCD4* cleavage product is involved in signalling pathways (Campbell et al., 2010). Push and blocking can be applied together; silencing of *TaHYD* and overexpression of *CrtB* produced higher levels of β-carotene in wheat (Zeng et al., 2015).

2.12.3. Metabolic sink capacity

Another strategy is by enlarging the capacity of the metabolic sink where the carotenoids are accumulating (Giuliano, 2017). The idea was originated from the overexpression of *OR* genes and their induction on the differentiation of chromoplast and β-carotene accumulation (Li and Van Eck, 2007). Genetic modification on the ABA pathway increased the number or size of plastids which consequently resulted in more accumulation of tomato lycopene (Galpaz et al., 2008). A cross between transgenic maize with astaxanthin content and high oil-maize genotype to increase the storage capacity of this component led to only a marginal increase of astaxanthin (Galpaz et

al., 2008). The application of metabolic sink strategy usually doubled the carotenoid content in plants (Giuliano, 2017).

2.12.4. Stability of carotenoids in the post-harvest period

In gold potato, the stability of carotenoids was increased over the cold storage period (Giuliano, 2017). Cereal carotenoids are vulnerable to oxidation and lipoxidation, but increasing vitamin E content and deleting the loci that code for lipoxygenase are effective ways to stabilize the carotenoids over storage time and/or during processing (Carrera et al., 2007; Che et al., 2016).

2.13. Natural variation

Many crops showed good variation of carotenoid types and content. Basically, this variation existed due to overexpression of structural genes in the pathway (Giuliano, 2017). Two genes, *CYC-b* and *LCY-e,* overexpressed during ripening and ultimately increased the level of both β and α-carotene (Ronen et al., 2000). Two recessive genes, *LCY-e* and *CHY1*, with lower expression levels resulted in the high accumulation of β-carotene (Harjes et al., 2008; Yan et al., 2010). Retrotransposon insertion in *ZEP* genes resulted in higher total carotenoids in potato and overexpression of *CHY2* selectively increased the content of zeaxanthin (Wolters et al., 2010). The accumulation of β-carotene in cassava root was the consequence of non-synonymous mutation in *PSY2* gene (Welsch et al., 2010).

There is a negative correlation between starch level and β-carotene level that makes breeding for quality improvement challenging in cassava (Beyene et al., 2018). In tomato (*Solanum lycopersicum* L.), two allelic mutants, pale yellow petal (*pyp*) *1-1* and *pyp1-2*, reduced yellow colour intensity in the petals and anthers due to the reduction of xanthophyll esters, which was related to the decrease of carotenoid content and abnormal chromoplast development. Xanthophyll esterification plays a key role in the sequestration of chromoplast carotenoids, and accumulation of these esters is critical for normal chromoplast development. Identification of mutant alleles responsible for *pyp1* phenotype was performed through the integration of next-generation sequencing and map-based positional cloning (Ariizumi et al., 2014). Two important loci Y1 and Y2 controlled the content of α and β-carotenes in carrot. Lines with *yy* y_2y_2 alleles had a high amount of these two components (Just et al., 2009). In the majority of crops, conventional breeding is an ideal approach to increase provitamin A level. However, in some crops such as potato, rice, and wheat using methods like gene transformation is necessary as a natural variation for β-carotene is very limited (Giuliano, 2017).

2.14. Gene expression analysis

Study on the correlation between carotenoid content in maize seed and transcription level of genes involved in carotenoid biosynthesis showed that specific gene family members are responsible for carotenoid content and composition during endosperm development (Li et al., 2008; Vallabhaneni et al., 2009; Vallabhaneni and Wurtzel, 2009). Toledo-Ortiz et al. (2010) revealed that phytochrome-interacting factor 1 (*PIF1*) and other transcription factors of the phytochrome interacting factor (*PIF*) are involved in down-regulation of carotenoid accumulation through repressing the gene encoding phytoene synthase (*PSY*) during etiolation. In tomato, the MADS box regulator of ripening, RIPENING INHIBITOR (RIN), interacts with the *PSY* promoter (Fujisawa et al., 2013). The interaction of Stay Green *SlSGR1* protein with the *PSY* enzyme in tomato regulates fruit lycopene and β-carotene accumulation (Luo et al., 2013). Kachanovsky et al. (2012) proposed that epistasis in tomato colour mutations regulates expression of *PSY1* by cis-carotenoids. Recent studies on transgenic carrot (*Daucus carota*) demonstrated that *DcLcyb1* that encodes for a *LCYB* enzyme is a key player in the carotenogenic pathway. Transcript changes in *DcLcyb1* levels produced not only changes in carotenoid accumulation but also in the thickness of storage root and expression of key endogenous carotenogenic genes (Moreno et al., 2013). The colour of watermelon flesh is mostly affected by lycopene. Gene expression analysis in watermelon showed that two genes, phytoene synthase 1 (WMU38667) and a lycopene beta cyclase (WMU41454), showed differential expression during fruit development (Guo et al., 2011).

Genome sequencing in tomato set the stage for fruit-specific gene neofunctionalization including phytoene synthase (*PSY*; The tomato genome consortium, 2012). Caroca et al. (2013) showed that chimeric expression elements could trigger significantly higher gene activity in chromoplasts than native elements in the plastid genome. Deep transcriptome sequencing in saffron (*Crocus sativus*) revealed a novel dioxygenase, carotenoid cleavage dioxygenase 2 (*CCD2*) that cleaves zeaxanthin, the presumed precursor of saffron apocarotenoids, both in *Escherichia coli* and in maize endosperm (Frusciante et al., 2014).

2.15. Carotenoid measurement

In a plant breeding program, a large number of samples must be assessed for different carotenoid components, but measurement of total carotenoids may be sufficient for initial screening (Rodriguez-Amaya and Kimura 2004; Kimura et al., 2007). One of the quick methods for estimation of total carotenoid is near infrared reflectance spectroscopy (NIRS), which is easy

and safe and can be performed with a very small amount of sample. This method was used for carotenoid measurement in maize (Brenna and Berardo, 2004), banana (Davey et al., 2009), and cassava (Sanchez et al., 2014). In comparison to NIRS, spectrophotometer is more time consuming and laborious for carotenoid measurement. In addition, samples need to be extracted using organic solvents. Laser photoacoustic spectroscopy (PAS), a photothermal approach, was used for analyzing carotenoid content in corn and sweet potato flours (Luterotti et al., 2011).

High-performance liquid chromatography (HPLC) with a reverse phase is the most prevalent method for carotenoid measurement in the last two decades. It does separation, detection, and quantification of various components which are dissolved in chemicals (Amorim-Carrilho et al., 2014; Rezaei et al., 2016). In addition, HPLC combined with mass spectrometry (HPLC-MS) has been used for carotenoid analysis (Slavin et al., 2009). Ultra high-performance liquid chromatography (UHPLC) has been developed with advanced column technology and instrumentation, which is faster and more sensitive than conventional HPLC. The results come in a narrower peak with higher resolution (Rivera and Canela-Garayoa, 2012b). This method has been used in different crops for carotenoid measurement such as corn (Rivera et al., 2013; Rivera and Canela, 2012a), durum wheat (Hung and Hatcher, 2011), tomato (Van Meulebroek et al., 2012; Li et al., 2012, 2013), and *Brassica oleraceae* (Kaulmann et al., 2014). Moreover, ultra-high performance supercritical fluid chromatography (UHPSFC) has been successfully used for carotenoid quantification in paprika oleoresin (Berger and Berger, 2013). There are several ionization approaches which are used for MS assay of carotenoids such as electron impact (EI), fast atom bombardment (FAB), matrix-assisted laser desorption/ionization (MALDI), electrospray (ESI), atmospheric pressure chemical ionization (APCI), atmospheric pressure photoionization (APPI), and atmospheric pressure solid analysis probe (ASAP; Enzell and Back 1995; van Breemen, 1995,1997; Dachtler et al., 2001; Aman et al., 2005).

Detection of esterified carotenoids in fruits and flowers is more complex and needs a combination of the methods mentioned above. For example, in the case of mango (Pott et al., 2003) and marigold flowers (Breithaupt et al., 2002), HPLC-APCI/MS was applied for the measurement of these types of carotenoids. According to Prasain et al., (2005) tandem mass spectrometry (MS/MS) is a selective and specific method for the analysis of oxidation product and zeaxanthin. A combination of nuclear magnetic resonance (NMR) and HPLC and off-line NMR can be used for analysis of geometric isomers (Dachtler et al. 2001; Glaser et al., 2003; Aman et al. 2005;

Tiziani et al., 2006). In tomato, identification of Z-isomers was successfully done by one- and two-dimensional NMR (Tiziani et al., 2006). In addition, there are more methods that have been proposed for carotenoid measurement including Raman spectroscopy (Bhosale et al., 2004), attenuated total reflectance infrared spectroscopy (ATR-IR; Baranska et al., 2006b), and fourier transform infrared spectroscopy (FTIR; Rubio-Diaz et al., 2011). Research showed that X-ray photoelectron spectroscopy (XPS) and time-of-flight secondary ion mass spectrometry (ToF-SIMS) methods are practical for major carotenoid determination in *Bixa orellana* seeds (Felicissimo et al., 2004).

2.16. Genetic analysis and breeding for carotenoid improvement

Genetic analysis showed that seed weight and carotenoid concentration are under the control of quantitative trait loci (QTL) in chickpea (Abbo et al., 2005). Pozniak et al. (2007) showed that phytoene synthase (*PSY1*) co-segregated with 7B QTL in durum wheat (*Triticum turgidum* L. var *durum*), which showed a correlation with the gene for endosperm colour. Analysis of QTL in maize showed that 6.6-27.2% variation of the seed carotenoid content was associated with the activity of the key enzyme *PSY1* (Chander et al., 2008a). The gene β-carotene hydroxylase1 (*HYD3*) was mapped close to the known QTL for β-carotene composition and this result was confirmed using association and linkage studies in maize (Yan et al., 2010). Crossing of two sweet potato lines with contrasting levels of β-carotene showed that 8 loci involved in production of β-carotene (Cervantes-Flores et al., 2011). There is an association between several QTLs including genes in the carotenoid pathway with the visual scores of relative orange endosperm colour intensity in maize (Chandler et al., 2013). An allelic polymorphism (SNP) in one of the two expressed phytoene synthase (*PSY*) genes in cassava (*Manihot esculenta*) was responsible for enhancing the flux of carbon through carotenogenesis, and as such resulted in the accumulation of carotenoids in storage roots. Consequently, cassava plants with over-expressed *PSY* transgene had yellow-fleshed and high-carotenoid roots (Welsch et al., 2010).

In potato, QTL analysis revealed that lines with dominant alleles of β-carotene hydroxylase 2 (*Chy2*) had higher carotenoid accumulation, especially β-xanthophylls (Wolters et al., 2010). Recent studies on maize carotenogenesis showed that two genes including *zep1*, responsible for zeaxanthin epoxidase expression, and *lut1* that encodes *CYP97C*, a cytochrome P450-type monooxygenase that is involved in lutein production through hydroxylation of ε-ring in zeinoxanthin, are significantly associated with grain carotenoid composition (Owens et al., 2014;

Quinlan et al., 2012). A genome-wide QTL and bulked transcriptomic analysis of potato (*Solanum tuberosum*) tuber carotenoid concentration resulted in the identification of a major QTL on chromosome 9 (Campbell et al., 2014).

CHAPTER 3. GENOME-WIDE ASSOCIATION STUDY OF CAROTENOID BIOSYNTHESIS IN CHICKPEA (*Cicer arietinum* L.) SEEDS

Preface

The main purpose of this chapter is to address first and second objectives, which is to identify the genomic regions associated with carotenoid concentration as well as to examine the environmental effects on carotenogenesis. To this end, a total of 172 chickpea accessions were genotyped using 50K Axiom® *CicerSNP* Array and different carotenoid components were assessed using high-performance liquid chromatography (HPLC). The chickpea collection were grown in Elrose, SK in 2015 and Limerick, SK in 2016 to evaluate the effects of genotype, environment, and interaction of genotype by the environment on five carotenoid components. The genome-wide association study (GWAS) revealed that the genes involved in the biosynthesis of apo-carotenoids and precursor of carotenoids are strongly associated with carotenoid concentration in chickpea. Moreover, the effects of genotype, environment, and genotype by environment were significant on most of the carotenoid components in chickpea.

3.1. Introduction

Plant carotenoids have several important roles such as antioxidant activity, provitamin A properties, photo protectant as well as being precursors for biosynthesis of plant hormones (Kermode, 2005; Moise et al., 2014). The carotenoid pigments β-carotene, lutein, violaxanthin and neoxanthin are predominantly found in plants. Among them carotenoids with β-ring (β-carotene, β-cryptoxanthin and α-carotene) are considered as provitamin A, which is converted to vitamin A or retinol through enzymatic cleavage activities in the human body (Stahl and Sies, 2005; Owens et al., 2014). The role of β-carotene is particularly important in many developing countries where deficiency of vitamin A is the leading cause of severe visual impairment and increases the risk of death especially for children and pregnant women (Haskell et al., 2005; West and Darnton-Hill, 2008).

In terms of production, chickpea (*Cicer arietinum* L.) is the second most important food legume after common bean (FAO, 2016). Chickpea seeds are rich in many important micro-nutrients and serve as the major source of protein and carbohydrate for human diets in many

developing countries, especially in Africa and South Asia (Saini and Knights, 1984; Wood and Grusak, 2007; Chibbar et al., 2010). Improving the nutritional quality of chickpea seeds could bring substantial impact on the health of millions of people.

Chickpea accessions with high total carotenoid concentration (25–35 μg g^{-1}) have been identified and can potentially be used for breeding for high carotenoid chickpeas (Ashokkumar et al., 2015). The main carotenoid in chickpea seed is lutein. The level of provitamin A carotenoids is high in cultivars with green cotyledon colour (Ashokkumar et al., 2015; Rezaei et al., 2016). The genetic basis for the variation in carotenoid concentrations in chickpeas is still unknown. Previously, four QTLs for β-carotene concentration and a single QTL for lutein concentration were reported in chickpea (Abbo et al., 2005). Recent developments in DNA sequencing and SNP genotyping techniques have provided ample single nucleotide polymorphism (SNPs) markers widely distributed across the chickpea genome (Deokar et al., 2014; Diapari et al., 2014). The presence of SNPs in both coding and non-coding regions, and their potential for high-throughput genotyping (Gupta et al., 2001), make them ideal markers for evolutionary, diversity and mapping studies (Kushanov et al., 2016) in various crops (Rafalski, 2002). Array-based SNP genotyping platforms such as Affymetrix Axiom™ and Illumina BeadChips™ arrays allow genotyping of thousands to millions of SNPs for routine genetic analysis, such as QTL mapping and GWAS without complex bioinformatic analysis (Hoffmanna et al., 2011; Xu et al., 2017b).

Recently, the Axiom® *CicerSNP* Array with 50K density was developed and used for QTL mapping in recombinant inbred line (RIL) populations of chickpea (Roorkiwal et al., 2017). The availability of a high-throughput genotyping method, allelic diversity, and a lack of the need to create a mapping population make the natural population, i.e., germplasm or crop accessions, a favorite choice for finding genomic regions for important traits through association analysis (Xu et al., 2017a). Association mapping has been routinely used for simultaneous evaluation of a wide range of alleles and traits in many species (Zhao et al., 2007; Diapari et al., 2014; Breseghello and Sorrells, 2006). Genome-wide association analysis combined with robust phenotyping across different environments can lead to the discovery of the genetic components behind important quantitative traits in plants (George and Cavanagh, 2015). High-performance liquid chromatography (HPLC) is an efficient and accurate method for assessment of carotenoids (Khachik et al., 1992). GWAS has been used for the identification of genes responsible for carotenoids in various crops. In cassava, SNPs within the vicinity of *Manes.01G124200.1* locus,

and candidate genes including the phytoene synthase, UDP-glucose pyrophosphorylase and sucrose synthase showed significant association with provitamin A content (Esuma et al., 2016; Rabbi et al., 2017). Twenty four genes responsible for biosynthesis and catabolism of carotenoids were identified through GWAS in wheat (Colasuonno et al., 2017). Association study revealed that both zeaxanthin epoxidase (*zep1*) and *lut1*, the gene that encodes the cytochrome P450-type monooxygenase, had a significant association with the carotenoid composition in maize (Owens et al., 2014). Genes involved in hydroxylation and cleavage reactions were considered ideal candidates for biofortification of carotenoids in maize (Suwarno et al., 2015). Thus far, no GWAS for carotenoids has been reported in chickpea. In chickpea, many natural inbred accessions are available. The inbreeding nature of chickpea resulted in patterns of polymorphism characterized by extensive haplotype structures that are critical for association study (Nordborg, 2000).

The main objective of this study was to identify the genomic regions associated with carotenoid concentration across 172 diverse chickpea accessions by GWAS. The results from this study can be applied to carotenoid biofortification efforts through molecular breeding to improve provitamin A content in this crop.

3.2. Materials and methodology

3.2.1. Plant materials

A total of 172 chickpea diverse accessions including germplasm and released cultivars of both desi and kabuli types were used for the analysis. The accessions were originated from Canada, Greece, India, Iran, Israel, Moldova, Morocco, Portugal, Russia, Syria, Spain, Tunisia, Turkey and the United States of America (Table A1). The genotypic panel varied for seed coat colour (brown, beige, green, white-off transparent and black) and cotyledon colour (yellow, orange and green). The chickpea accessions were grown at Elrose, SK in 2015 and Limerick, SK in 2016. At each location, each accession was seeded in 1 m × 3 m plot. The plots were arranged as a randomized complete block design (RCBD) with three replications.

Agronomic traits including days to flowering, reaction to ascochyta blight, plant height, days to maturity and 1000 seed weight were recorded for each plot. The number of days between emergence and the date when at least 50% of plants within a plot had open flowers was recorded as days to flowering. Ascochyta blight disease severity was scored using a 0 to 9 scale with 0 and 9 representing no symptom and dead plants, respectively (Chongo et al., 2004). Plant height was

measured in cm based on the average of three measurements per plot from the ground surface to the tip of the main stem. Days to maturity was calculated based on the difference between the date when more than 50% of the pods within a plot turned colour, and the seeding date. Thousand-seed weight was determined by measuring and converting the weight of 200 seeds (12% moisture content) using an electronic balance.

3.2.2. Protein, amino acids and fat assessment

Chickpea seeds, grown in Elrose 2015, were ground once through a UDY grinder (Fort Collins CO) with a 1.0 mm sieve and 200 mg of fine powder were analyzed using the LECO (Model FP628, LECO Corporation, Saint Joseph, MI) according to AACC International Method 46-30.01. The results were expressed in percent nitrogen and multiplied by a correction factor of 6.25, specific to pulses in general (Jones, 1941), to obtain the percent protein.

For amino acid extraction, 100 mg of fine powder was mixed with 7 ml of 50% ethanol in a 10 cm × 1.5 cm screw-top tube. The tubes were placed in a Labquake shaker in a 50 ºC oven for 20 minutes. The tubes were centrifuged at 1,300 x g for 20 minutes. A 0.5 ml aliquot was taken and assessed according to the procedure as indicated in the Phenomenex EZ: faast™, amino acid analysis kit, for measurement of 18 amino acids (µg g^{-1}). Two µl of the extract were injected into an Agilent gas chromatograph (Model 7890A, Santa Clara, CA) equipped with a Zebron ZB-AAA capillary column (10 m x 0.25 mm) and a flame ionization detector at a split ratio of 1:15. The injection temperature was set at 250ºC. The initial oven temperature was set at 110 ºC and increased to 320 ºC at 32 ºC $minute^{-1}$.

Two grams of fine powder were dried in an oven at 102ºC for 3 hours. The loss in weight is taken as the amount of moisture loss upon drying and is expressed as % moisture. The dried powder was extracted in an ANKOM XT15 Fat Extractor (ANKOM Technology, Macedon, NY) with hexane as the solvent and set at 105ºC for two hours. The loss in weight of the sample is taken as the amount of fat extracted and is expressed as % fat.

3.2.3. Carotenoid measurement

The carotenoid extraction process used 100 mg of fine powder from whole chickpea seeds (10% moisture content) collected from the field in Elrose (2015) and Limerick (2016). The time between harvest and carotenoid measurement was the same for both years. All seeds were harvested late in September and carotenoid analysis was done in January of the following year. The chickpea seeds were kept in room temperature with average 22 °C air temperature and 45%

relative humidity. The storage condition was similar for all samples from both years. The sample was premixed with 400 µl of 1:1 methanol and DCM (dichloromethane). The mixture was, then, centrifuged for 15 minutes at 11,000 rpm. The supernatant was transferred to a new tube and 400 µl of 100% acetonitrile was added and centrifuged for 5 minutes at 11,000 rpm. To minimize carotenoid oxidation, the solution was mixed with 0.1% BHT (butylated hydroxytoluene). Chromatography was done using the Agilent 1200 LC system with Chemstation software (Agilent Technologies, Santa Clara, CA, USA). Separation was done on Prodigy 5 µm (250 x 4.60 mm) column with the mobile phase 58:20:22 acetonitrile/dichloromethane/methanol flowing at 0.8 ml minute^{-1}. 100 µl from each sample was injected and run for 45 minutes. Detection of various components was done using a photodiode array detector monitoring at a 450 nm wavelength (Rezaei et al., 2016).

Five standards including lutein, violaxanthin (ChromaDex, Irvine, CA, USA), zeaxanthin, β-carotene, and β-cryptoxanthin (95 % purity; Sigma-Aldrich Canada, Oakville, ON) were used to make linear standard curves as described by Ashokkumar et al. (2014). The regression equation for the calibration of each component was obtained as follows: violaxanthin ($y = 11.6x - 16.95$, $R^2 = 0.992$), lutein ($y = 10.5x + 27.88$, $R^2 = 0.999$), zeaxanthin ($y = 8.1x + 5.81$, $R^2 = 0.995$), β-cryptoxanthin ($y = 40.02x + 56.2$, $R^2 = 0.999$) and β-carotene ($y = 15x + 47.63$, $R^2 = 0.999$); y denotes peak area and x indicates concentration (µg g^{-1} ml^{-1}). UV-visible spectra analysis and comparison of carotenoids retention time with the authentic standard was used for carotenoid determination (Ashokkumar et al., 2014). The retention time was 3.8, 4.5, 5.6, 9.3 and 22.3 min for violaxanthin, lutein, zeaxanthin, β-cryptoxanthin and β-carotene, respectively (Rezaei et al., 2016). Three biological replicates with two injections as technical repeats by HPLC were used for each sample. The results were converted to µg g^{-1} as carotenoid concentration.

3.2.4. Statistical analysis

Analysis of variance components were determined using PROC MIXED of SAS version 9.3 for Windows. Homogeneity of variance for each trait was assessed with Levene's test prior to analysis and heterogeneous variances were analyzed with the repeated statement of PROC MIXED. Cultivars were considered as a fixed effect and site-year (locations and year) and replications (blocks) within each site-year were considered as a random effect.

The ratio of genetic and phenotypic variances was used for the determination of broad sense heritability ($H^2 = \sigma^2_g / \sigma^2_p$). The phenotypic variance was calculated as $\sigma^2_p = \sigma^2_g + \sigma^2_{ge} / m +$

$\sigma^2_{er} / (m \times b)$, where σ^2_{ge} and σ^2_{er} are estimates for genotype by site-year interaction and residual error variances, m is the number of site-years and b is the number of replications. Pearson's correlation was estimated among different traits with the SAS Proc Corr statement.

3.2.5. Genotyping

DNA was extracted from the young leaves of each of the 172 chickpea accessions following the protocol of DNeasy® Plant Mini Kit (QIAGEN) in the Pulse Crop Molecular Breeding Lab at the Department of Plant Sciences, University of Saskatchewan. All the DNA samples were sent to the Genomics Lab at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) for genotyping using a 50K Axiom® *CicerSNP* Array (Roorkiwal et al., 2017). The array was created based on the SNPs derived from resequencing data of 429 chickpea lines. Specific criteria such as flanking region without SSR and indel, biallelic SNPs, minor allele frequency of 0.05, CG content ranging from 40%-70%, SNPs with quality score ≥ 30 were considered for designing this chip (Roorkiwal et al., 2017).

3.2.6. Population structure

Population structure of the 172 chickpea accessions was analyzed using the distance-based hierarchical approach of Bayesian based model clustering and principal component analysis (PCA). In Bayesian clustering, the accessions were considered as an admixture population. The analysis of subpopulations of the accessions was conducted using STRUCTURE software (Pritchard et al., 2000). Delta K (ΔK) analysis was conducted using SCRIPT in STRUCTURE HARVESTR (Earl and vonHoldt, 2012) and the number of subpopulations was estimated according to the highest ΔK value (Evanno et al., 2005). PCA analysis was performed using the function "svd" in R (R Development Core Team, 2016) to compare the number of subpopulations within the 172 accessions derived from this method with the number of subpopulations from structure analysis. Missing markers were replaced with the numeric genotype mean for that marker to perform PCA.

3.2.7. Linkage disequilibrium (LD) decay

The LD estimate (r^2) was calculated using the Plink program for both linked (same chromosome) and unlinked (different chromosome) markers. Based on the results from linked loci, the r^2 values were plotted against the genetic distance and a smooth non-linear regression was created in R (R Development Core Team, 2016). The critical r^2 value was measured based on the

95th percentile distribution of unlinked markers. The LD decay average was considered as the intersection of the regression and the critical line across the chickpea accessions.

3.2.8. Association analysis

A total of 24,095 SNPs with minor allele frequency (MAF) > 5% was used for the association analysis. Linkage disequilibrium (LD) was calculated using TASSEL software (Bradbury et al., 2007). The mixed linear model (MLM) was used for the association test using related coefficient between chickpea accessions (K matrix) as a random effect and principal component analysis (PCA) as a fixed effect. To minimize the type I error, false discovery rate (FDR) test (Benjamini and Hochberg, 1995) was applied to obtain the adjusted P value.

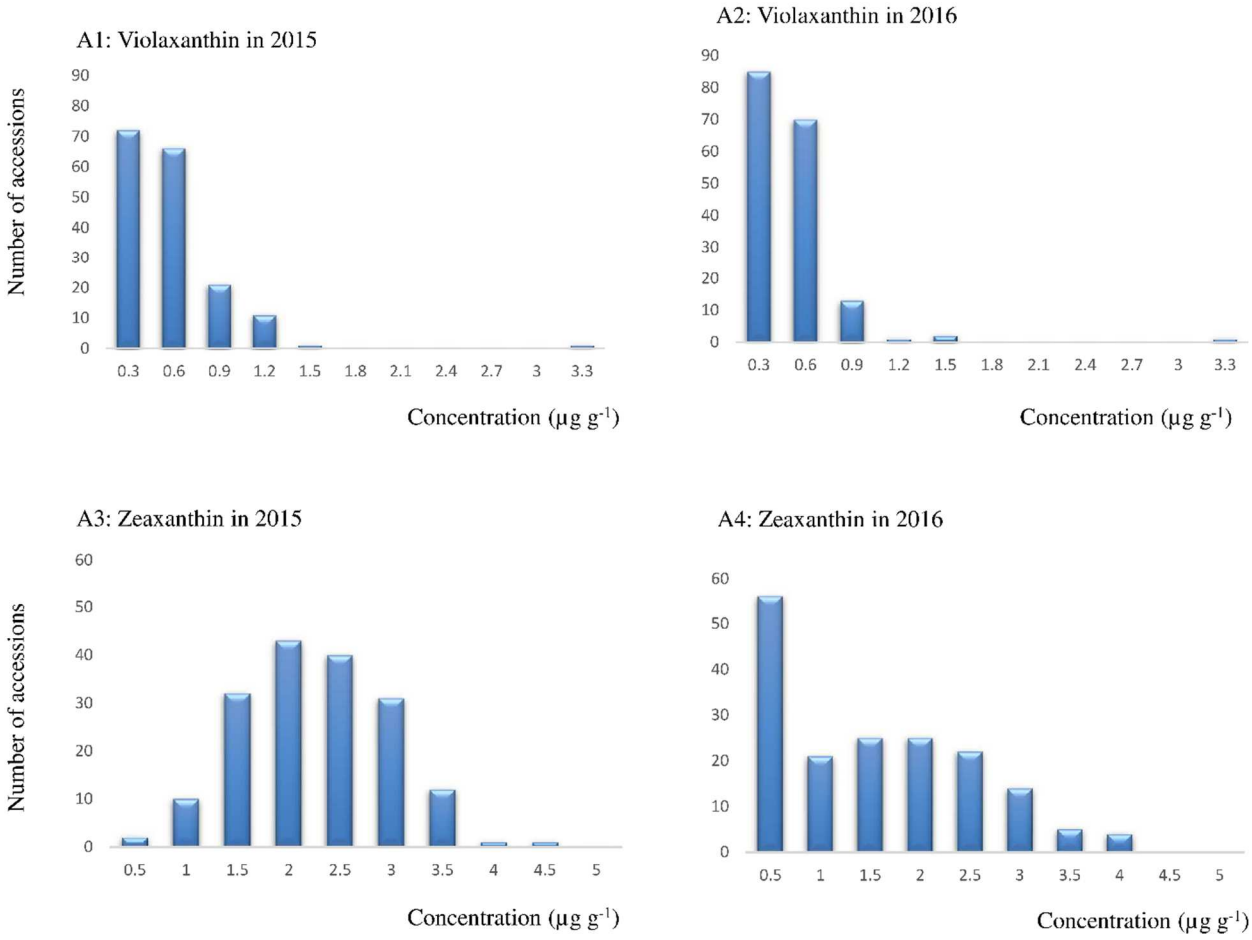
To find the candidate genes associated with carotenoid concentration we used "window" approach with 200 kb sliding. This is the most common method to assign SNP markers to genes (Petersen et al., 2013). The window size can vary up to 500 kb distance from a SNP marker (Wang et al., 2007). Here we used a fixed 200 kb window which was satisfactory to find SNPs assigned to carotenoid genes in GWAS.

3.3. Results

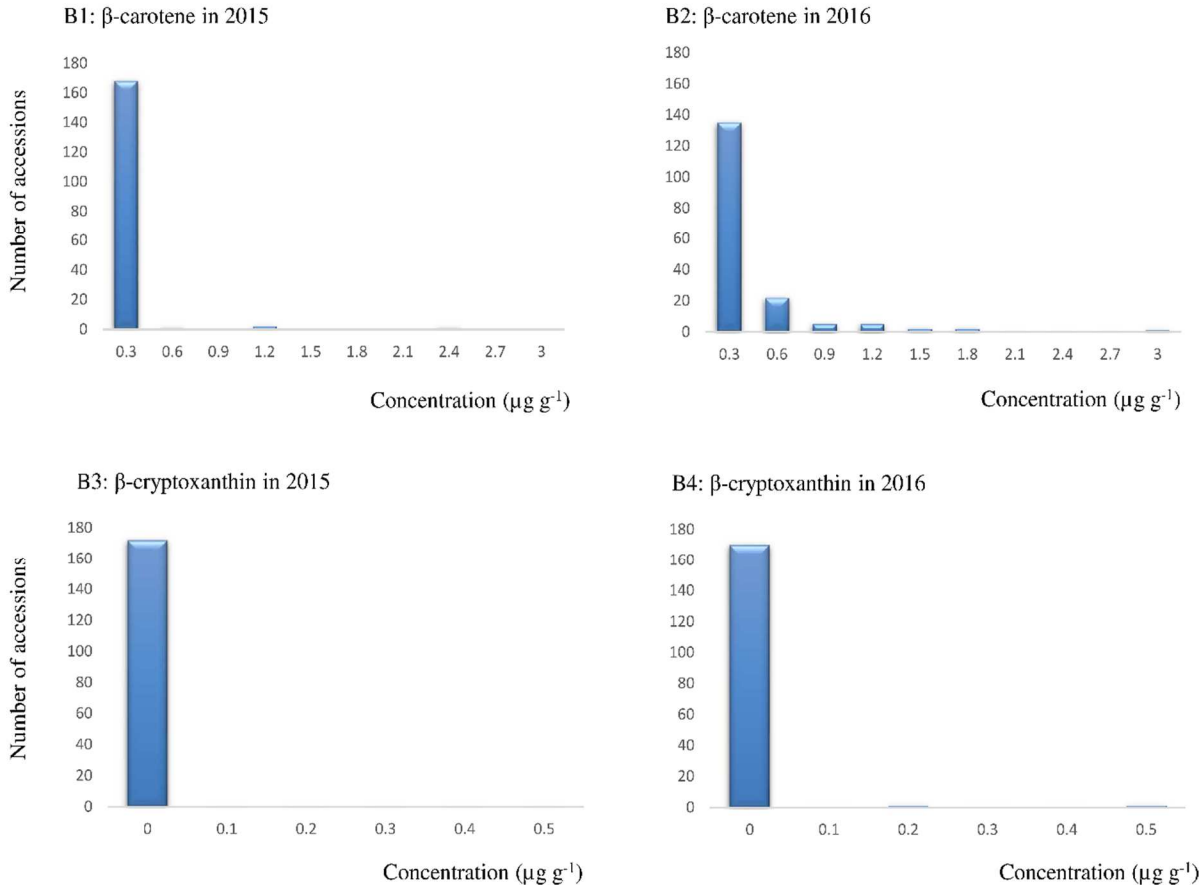
3.3.1. Carotenoid measurement

Based on cotyledon colour variation the chickpea accessions can be divided into three groups: green, yellow, and orange colour. The major carotenoid component in accessions panel was lutein followed by zeaxanthin. The concentrations of violaxanthin, β-cryptoxanthin, and β-carotene varied significantly among the accessions. Violaxanthin was present in almost all accessions, but β-cryptoxanthin and β-carotene were present mainly in accessions with green or orange cotyledon colour (Table A2 and 3). For example, in 2015 only 9 chickpea accessions out of 172 were observed with a detectable β-carotene level and in 2016 the number increased up to 40 accessions (Figure 3.1). Based on the average data in these two years for different carotenoids, the concentration range for each component are as follows: 3.5-28.2 µg g^{-1} for lutein, 0-3.04 µg g^{-1} for violaxanthin, , 0.27-2.84 µg g^{-1} for zeaxanthin, 0 for β-cryptoxanthin and 0-2.5 µg g^{-1} for β-carotene. CDC Jade, a green cotyledon desi cultivar with 36.6 µg g^{-1} total carotenoid concentration was the highest in the panel, while the line 846-1, a yellow desi accession, had the lowest total carotenoid concentration at 3.4 µg g^{-1}. Overall, the concentration of each carotenoid component was higher in 2016 at Limerick than in 2015 at Elrose (Figure 3.1 and Tables A2, 3).

A: Distribution of violaxanthin (A1-A2) and zeaxanthin (A3-A4) measured by HPLC in 172 chickpea accessions grown in Elrose, 2015 and Limerick, 2016



B: Distribution of β-carotene (B1-B2) and β-cryptoxanthin (B3-B4) measured by HPLC in 172 chickpea accessions grown in Elrose, 2015 and Limerick, 2016



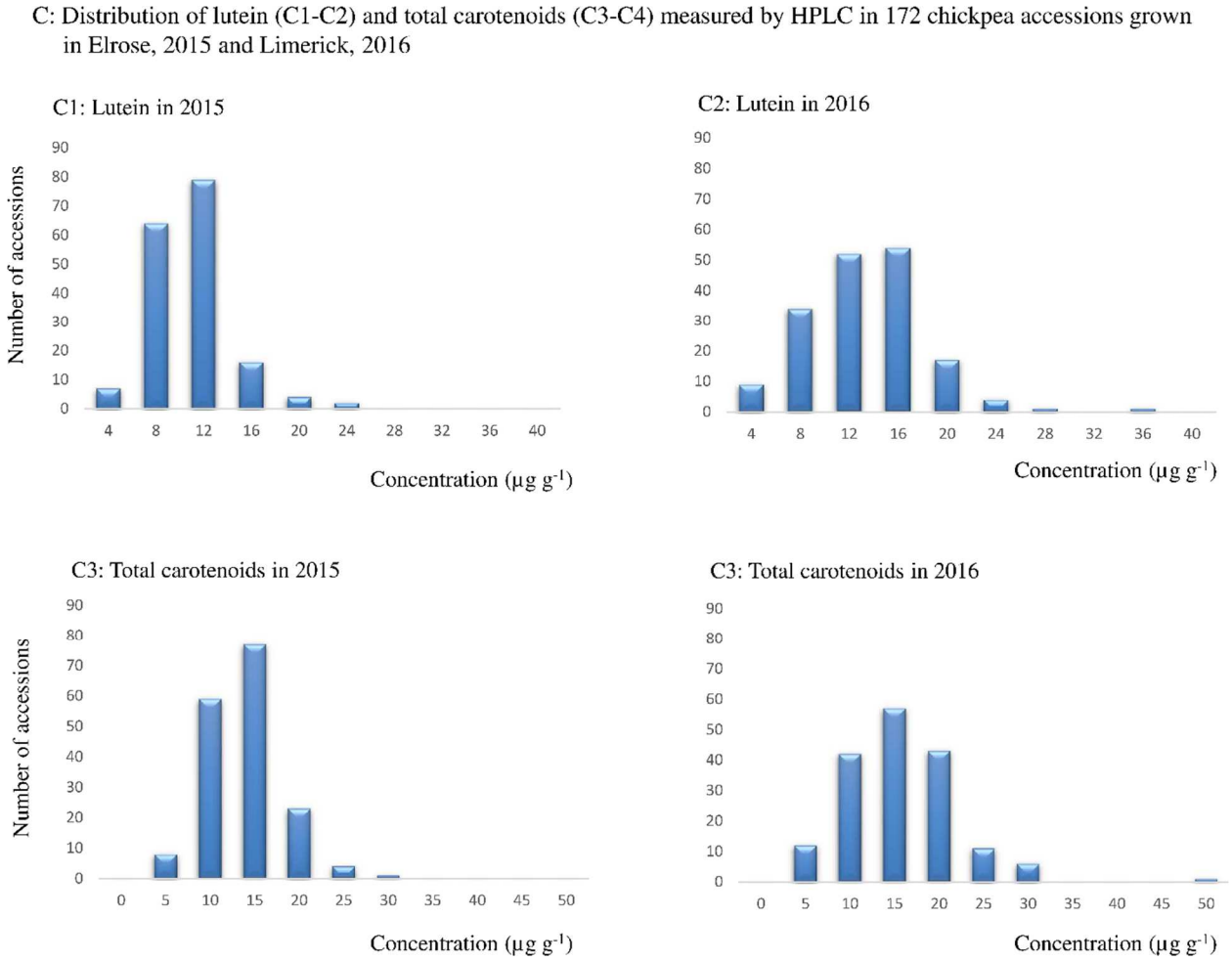


Figure 3.1. The distribution of carotenoid components including violaxanthin (A1-A2), zeaxanthin (A3-A4), β-carotene (B1-B2), β-cryptoxanthin (B3-B4), lutein (C1-C2), and total carotenoid (C3-C4) concentration (µg g^{-1}) which were measured using HPLC method across 172 chickpea accessions in Elrose, 2015 and Limerick, 2016.

3.3.2. The interaction of genotypes and environments for carotenoids and agronomic traits in genotypic panel

There were significant variations across 172 genotypic panel of the two years' combined data. The flowering time ranged from 33 (early flowering) to 42 (late flowering) days after seed sowing. Reaction to ascochyta blight varied from 2.3 (moderate resistant) to 8.5 (highly susceptible). Plant height varied from 24 cm (shortest) to 76 cm (tallest) and days to maturity ranged from 99 (early maturity) to 117 (late maturity).

ANOVA showed that the effects of genotype (G), site-year (E) and genotype by site-years (G × E) were significant for all traits across the two site-years except for β-cryptoxanthin and plant height. The effect of site-year was not significant for plant height, but the effects of genotype and interaction of genotype by site-year were significant. The effects of genotype, site-year and the interaction of genotype by site-year were not significant for β-cryptoxanthin concentration (Table 3.1). The highest (0.79) and lowest (0.30) broad sense heritability estimates were found for violaxanthin and zeaxanthin, respectively, while moderate heritability was observed for lutein (0.48), β-carotene (0.59) and total carotenoid (0.51) concentrations. The heritability estimates for 1000 seed weight (0.78) and plant height (0.83) were high, followed by moderate (0.55) for reaction to ascochyta blight, and relatively low (0.13) for maturity (Table 3.2).

Table 3.1. ANOVA for concentration (µg g^{-1}) of violaxanthin, lutein, zeaxanthin, β-cryptoxanthin, β-carotene and total carotenoids across 172 chickpea accessions grown in Elrose (2015) and Limerick (2016).

Source	df	Violaxanthin	Lutein	Zeaxanthin	β-cryptoxanthin	β-carotene	Total carotenoids
G	171	12.48^{***}	3.95^{***}	3.23^{***}	0.99^{ns}	4.84^{***}	4.33^{***}
E	1	28.38^{***}	96.39^{***}	105.97^{***}	1.72^{ns}	50.24^{***}	36.59^{***}
G × E	171	2.62^{***}	2.03^{***}	2.26^{***}	0.99^{ns}	1.97^{***}	2.11^{***}
Error	688						
Total	1031						
Variance components							
σ^2_{er}		0.041	17.36	1.28	0.001	0.08	25.09
σ^2_g		0.06 (50%)	5.58 (17%)	0.20 (9%)	0	0.03 (25%)	9.27 (20.3%)
σ^2_e		0.002 (1.5%)	3.09 (9.5%)	0.25 (11.2%)	2×10^{-6}	0.007 (4.9%)	1.59 (3.5%)
σ^2_{ge}		0.02 (16%)	5.98 (18.5%)	0.53 (23.5)	0	0.02 (17.1%)	9.35 (20.5%)
H^2		0.79	0.48	0.3		0.59	0.51

Note: df, degree of freedom; G, E and G × E are genotype, site-year and genotype by site-year interaction respectively; σ^2_g, σ^2_{ge}, σ^2_e, σ^2_{er} estimates of genotypic, genotype by site-year interaction, site-year, and error variance, respectively. cv, coefficient of variation, *H*, heritability. *, **, and *** indicate significant difference at $P \leq 0.05$, 0.01, and 0.001, respectively. ns, non-significant.

Table 3.2. ANOVA for agronomic traits including ascochyta blight score, plant height, maturity and 1000 seed weight across 172 chickpea accessions grown in Elrose (2015) and Limerick (2016).

Source	df	Ascochyta blight	Plant height	Maturity	1000 Seed weight
G	171	5.4^{***}	6.46^{***}	1.98^{***}	27.93^{***}
E	1	1489.6^{***}	0.08^{ns}	1462.24^{***}	5373.53^{***}
G × E	171	2.42^{***}	1.38^{**}	1.71^{***}	4.6^{***}
Error	688				
Total	1031				
Variance components					
σ^2_{er}		0.37	33.53	16.92	555.01
σ^2_g		0.19 (10.2%)	29.32 (42.7%)	0.76 (1%)	2321.48 (23.4%)
σ^2_e		1.10 (58.9%)	0	48.32 (68%)	6175.95 (62.4%)
σ^2_{ge}		0.18 (9.9%)	4.77 (6.9%)	4.15 (5.8%)	731.03 (7.3%)
H^2		0.55	0.78	0.13	0.83

Note: df, degree of freedom; G, E and G × E are genotype, site-year, and genotype by site-year interaction, respectively; σ^2_g, σ^2_{ge}, σ^2_e, σ^2_{er} estimates of genotypic, genotype by site-year interaction, site-year, and error variance, respectively. *H*, heritability. *, **, and *** indicate significant difference at $P \leq 0.05$, 0.01, and 0.001, respectively. ns, non-significant.

3.3.3. Correlation between carotenoids and agronomic traits

In 2015 at Elrose, violaxanthin concentration was positively correlated with lutein (r=0.35, P<0.001), β-carotene (r=0.49, P<0.001), and total carotenoids (r=0.41, P<0.001). Lutein showed significant positive correlation with zeaxanthin (r=0.20, P<0.01) and β-carotene (r=0.23, P<0.01) and negative correlation with seed weight (r= -0.23, P<0.01). The correlation between zeaxanthin, total carotenoids (r=0.83, P<0.001), plant height (r=0.18, P<0.05) and maturity (r=0.25, P<0.001) were positive and significant. β-carotene showed positive correlation with total carotenoids (r=0.39, P<0.001) and negative correlation with maturity (r= -0.15, P<0.05) and seed weight (r= -0.16, P<0.05). Like lutein, total carotenoids was negatively correlated with seed weight (r= -0.2, P<0.01). In addition, a negative correlation was observed between ascochyta blight disease score and maturity (r= -0.26, P<0.001), whereas a positive correlation was discovered between height and maturity (r= 0.19, P= P<0.01). Moreover, maturity and seed weight were positively correlated (r= 0.26, P<0.001) in 2015. (Table 3.3 A). In 2016 at Limerick, violaxanthin concentration was positively correlated with lutein (r= 0.52, P<0.001), zeaxanthin (r= 0.36, P<0.001), β-carotene (r= 0.53, P<0.001), and total carotenoids (r= 0.58, P<0.001) but negatively correlated with seed weight (r= -0.23, P<0.01). Lutein showed positive correlation with zeaxanthin (r= 0.52, P<0.001), β-

carotene (r= 0.55, P<0.001), total carotenoids (r= 0.97, P<0.001), and ascochyta disease score (r= 0.22, P<0.01). Positive correlations were observed between zeaxanthin and β-carotene (r= 0.27, P<0.001), total carotenoids (r= 0.67, P<0.001), and seed weight (r= 0.2, P<0.01). The concentration of two provitamin A carotenoids β-carotene and β-cryptoxanthin were positively correlated in 2016 (r= 0.18, P<0.05), and β-carotene was highly correlated with total carotenoids (r= 0.59, P<0.001). Ascochyta disease had positive correlation with total carotenoids (r= 0.19, P<0.05) and maturity (r= 0.15, P<0.05), but was negatively correlated with seed weight (r= -0.43, P<0.001; Table 3.3 B).

Table 3.3. Pearson correlation coefficients for carotenoid components including violaxanthin, lutein, zeaxanthin, β-cryptoxanthin, β-carotene and total carotenoid concentration (µg g^{-1}) as well as agronomic traits such as ascochyta blight scores, plant height, maturity and 1000 seed weight across 172 chickpea accessions grown in Elrose in 2015 (A) and Limerick in 2016 (B).

A	2	3	4	5	6	7	8	9 (1000 seed weight)
Violaxanthin (1)	0.35^{***}	0.11^{ns}	0.49^{***}	0.41^{***}	-0.13^{ns}	0.02^{ns}	0.04^{ns}	-0.1^{ns}
Lutein (2)		0.80^{***}	0.36^{***}	0.99^{ns}	-0.06^{ns}	0.05^{ns}	0.12^{ns}	-0.23^{**}
Zeaxanthin (3)			0.07^{ns}	0.83^{***}	-0.01^{ns}	0.18^{*}	0.25^{***}	0.003^{ns}
β-carotene (4)				0.39^{***}	0.07^{ns}	-0.005^{ns}	-0.15^{*}	-0.16^{*}
Total carotenoids (5)					-0.05^{ns}	0.07^{ns}	0.13^{ns}	-0.2^{**}
Ascochyta (6)						0.08^{ns}	-0.26^{***}	-0.12^{ns}
Plant height (7)							0.19^{**}	0.05^{ns}
Maturity (8)								0.26^{***}

Note: *, **, *** are significant at P ≤ 0.05, 0.01 and 0.001, respectively; ns, non-significant.

B	2	3	4	5	6	7	8	9	10 (1000 seed weight)
Violaxanthin (1)	0.52^{***}	0.36^{***}	0.11^{ns}	0.53^{***}	0.58^{***}	0.06^{ns}	-0.11^{ns}	-0.04^{ns}	-0.23^{**}
Lutein (2)		0.52^{***}	0.01^{ns}	0.55^{***}	0.97^{***}	0.22^{**}	0.06^{ns}	0.01^{ns}	-0.06^{ns}
Zeaxanthin (3)			0.002^{ns}	0.27^{***}	0.67^{***}	-0.001^{ns}	0.16^{*}	0.06^{ns}	0.2^{**}
β-cryptoxanthin (4)				0.18^{*}	0.03^{ns}	0.12^{ns}	-0.02^{ns}	-0.04^{ns}	-0.13^{ns}
β-carotene (5)					0.59^{***}	0.11^{ns}	0.01^{ns}	0.01^{ns}	-0.13^{ns}
Total carotenoids (6)						0.19^{*}	0.08^{ns}	0.02^{ns}	-0.03^{ns}
Ascochyta (7)							-0.02^{ns}	0.15^{*}	-0.43^{***}
Plant height (8)								0.02^{ns}	0.02^{ns}
Maturity (9)									-0.05^{ns}

Note: *, **, *** are significant at P ≤ 0.05, 0.01 and 0.001, respectively; ns, non-significant.

3.3.4. Population structure

A total of 50,590 SNP markers arranged as an Axiom® *CicerSNP* Array were used for genotyping of 172 chickpea accessions, however, after filtering the SNPs with MAF > 5% , only 24,095 high quality SNPs were used for genetic analysis. To evaluate the genetic diversity across the chickpea panel, the population structure and the number of subpopulations were determined using 529 unlinked markers using STRUCTURE 2.3.4 (Pritchard et al., 2000). Ten Ks were assessed for population structure analysis, and finally the K2 with highest ΔK was selected for further analysis (Figure 3.2). Based on K2, 119 accessions (116 kabuli and 3 desi) were grouped in subpopulation I and 53 accessions (40 desi and 13 kabuli) were grouped in subpopulation II. The results of population structure analysis with K2 are shown in Figure 3.3.

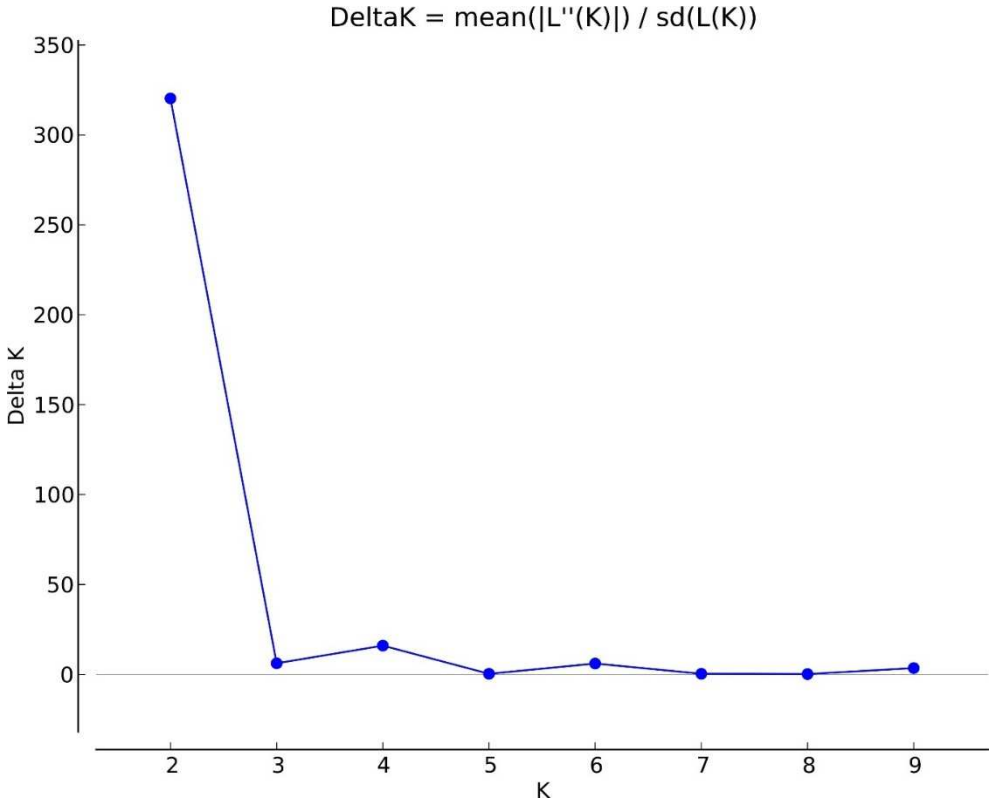


Figure 3.2. Delta K analysis for the structure of 172 chickpea accessions showing the highest probability with two subpopulations (K = 2).

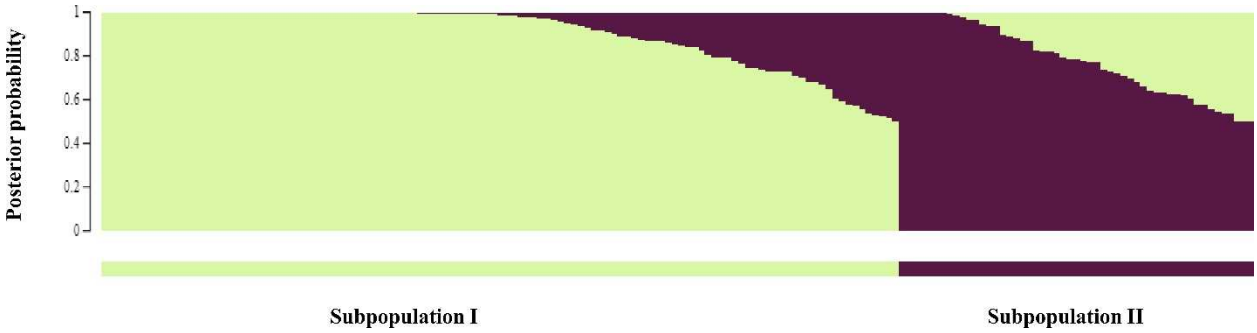


Figure 3.3. Population structure of 172 chickpea accessions based on K2 representing two subpopulations. Subpopulation I (light green) contains 119 accessions with the majority of kabuli type and subpopulation II (dark purple) contains 53 accessions with the majority of desi type. The Y-axis represents posterior probabilities and the X-axis represents different colours corresponding to each subpopulation.

Moreover, the principal component analysis (PCA) confirmed that there are two major subpopulations within the 172 accessions representing kabuli and desi types. Five principal components were used for the association analysis. The proportion of variance explained by each PC are as follows: PC1=24.2%, PC2=17.3%, PC3=8.5%, PC4=6.6% and PC5=4.9%. The first two components separated the two subpopulations of chickpea accessions (Figure 3.4).

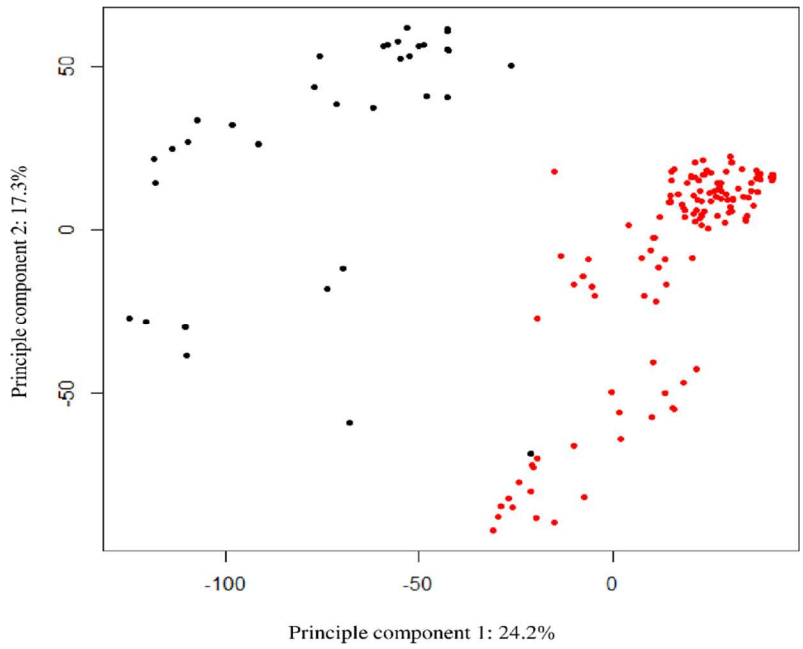


Figure 3.4. The plot of the first two principal components generated using 24,095 SNP markers on 172 chickpea accessions. The accessions were divided into two groups kabuli and desi types which are shown as black and red dots, respectively.

In addition, the neighbor joining (NJ) tree was constructed using 24,095 SNP markers to assess the genetic diversity of the 172 accessions. The analysis resulted in three clusters including 114 accessions in the first cluster with the majority of kabuli type, 28 accessions in the second cluster with the majority of desi type, and 30 accessions in the third cluster which consisted of both kabuli and desi types (Figure 3.5).

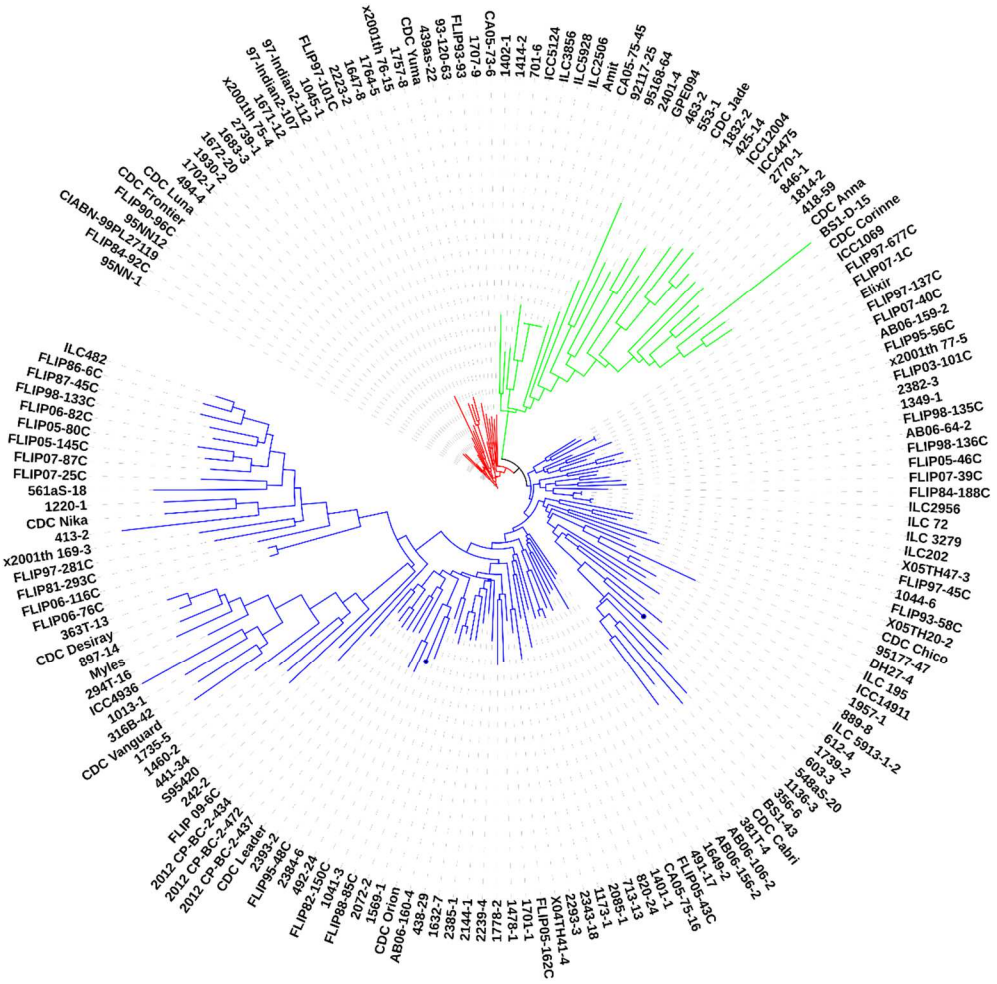


Figure 3.5. Neighbour joining tree was created using 24,095 SNP markers generated from 50K Axiom® *CicerSNP* array. The tree reveals three major clusters including 114 accessions in the first cluster with the majority of kabuli type (blue clade); 28 accessions in the second cluster with the majority of desi type (green clade) and 30 accessions in the admixed cluster (red clade).

3.3.5. Decay of linkage disequilibrium (LD)

The LD estimate in the genotypic panel showed that the LD starts to decay at 0.6 Mb corresponding with the r^2 value of <0.3 (Figure 3.6). This value indicated the average of LD decay across all 8 chickpea chromosomes that reflected the average size of LD block within the chickpea genome.

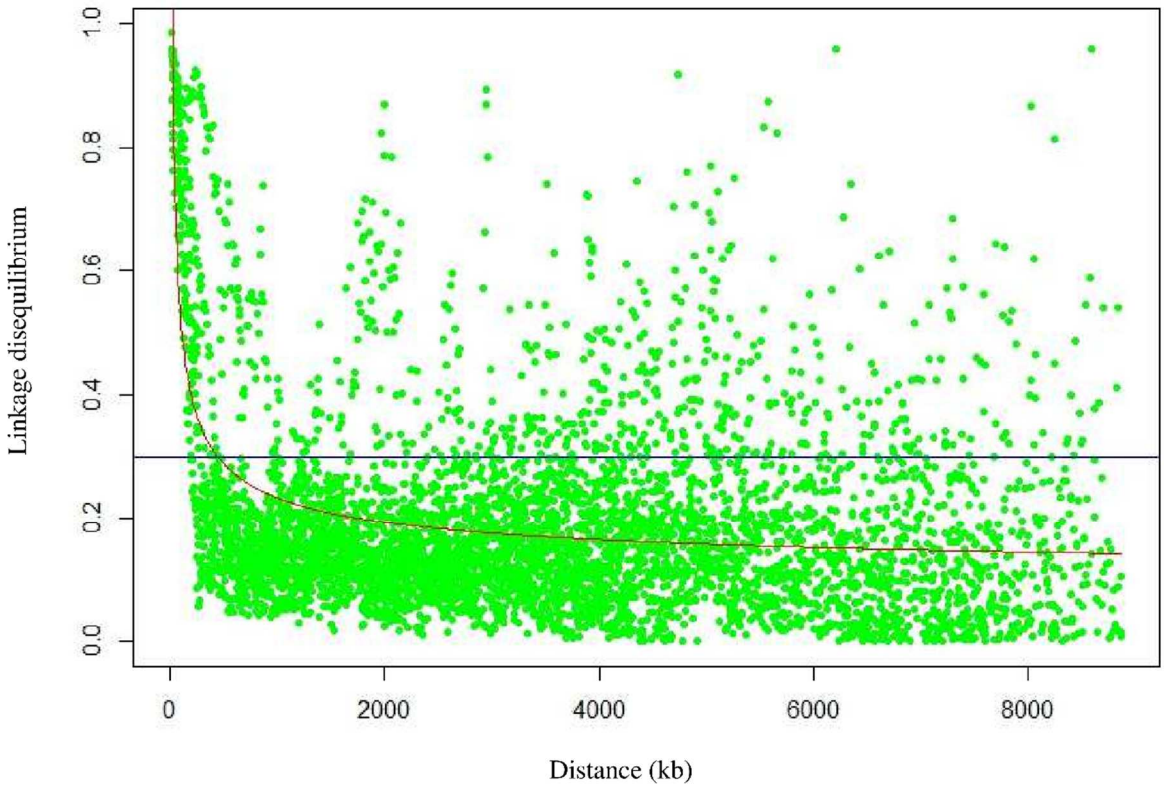


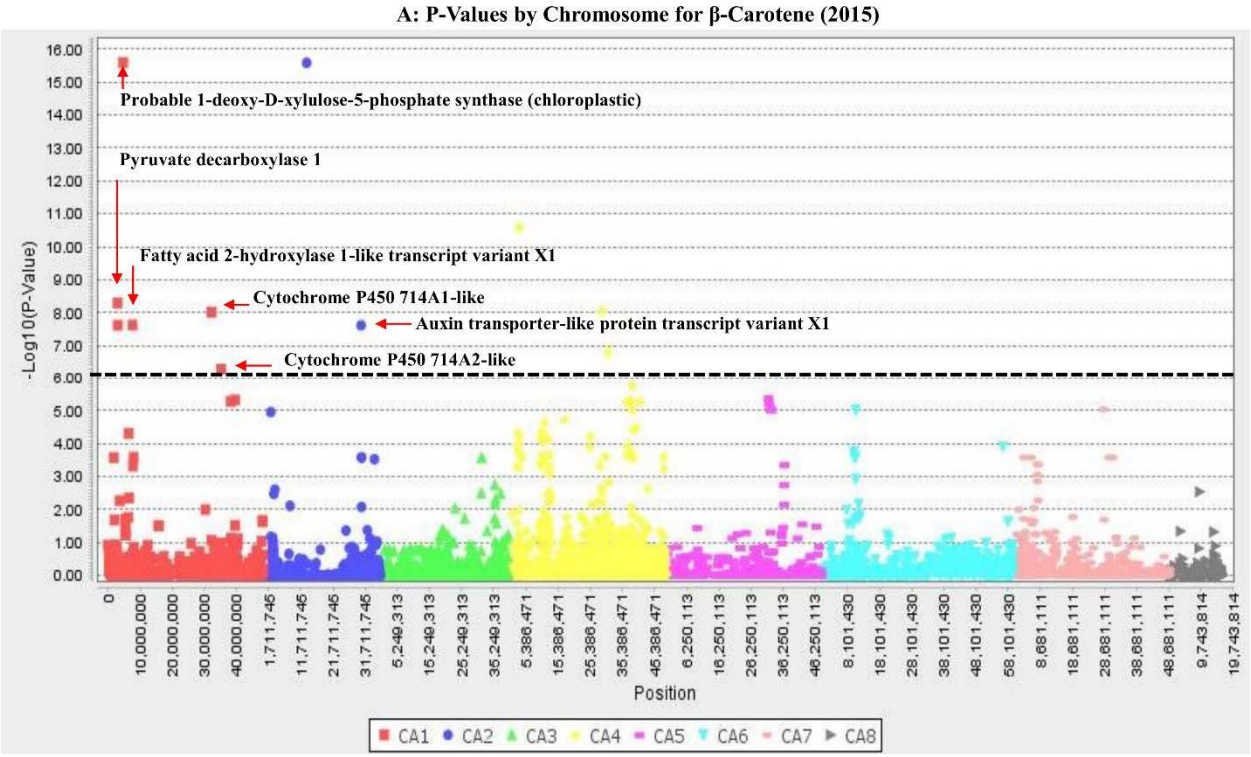
Figure 3.6. Decay of the linkage disequilibrium (LD) across the chickpea genome. The scatter plot shows the r^2 value of linked markers against the physical distance (kb). The intersection of smoothed non-linear regression line (red) and critical line (black) shows the point that average LD starts to decay at 0.6 Mb.

3.3.6. Association analysis

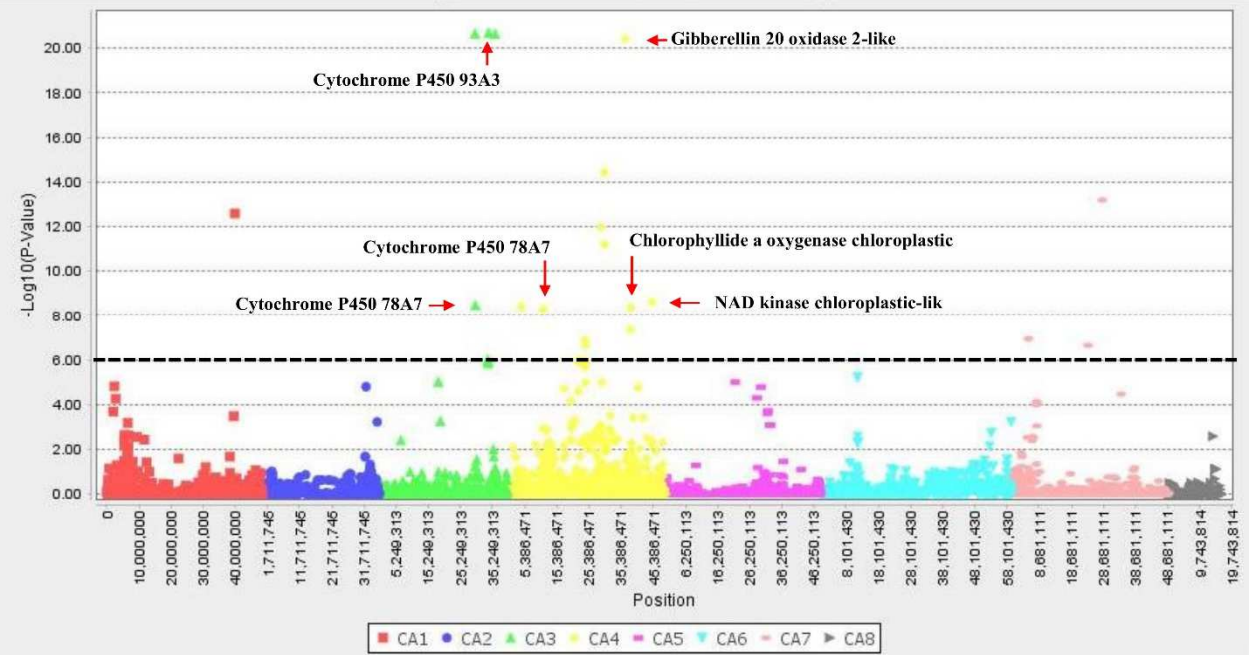
The association analysis between the SNP markers and the carotenoid components among 172 accessions was done using TASSEL software (Bradbury et al., 2007). The *P*-values from the association analysis were adjusted using the Benjamini and Hochberg (1995) approach by controlling the false-discovery rate (FDR) at 5%. The results of the association between the SNP markers and the carotenoid components were displayed as Manhattan plots with chromosomal

location is shown along the X-axis and the –log10 *P* values of each SNP are displayed on the Y-axis. The analysis resulted in the identification of 120 SNP markers that had significant association with concentration of different carotenoids in the accessions across the two environments. Further analysis including identification of potential candidate genes showed that 17 SNP markers were linked, either upstream or downstream, of the genes involved in carotenoid metabolism (Table 3.4 A). Five SNPs on chromosome one were significantly associated with β-carotene concentration from the 2015 trial at Elrose, SK. The first marker, Affx_123277641 (R^2 = 25%), was located in the upstream of pyruvate decarboxylase 1, the second marker Affx_123269438 (R^2 = 54%) was located in the downstream of 1-deoxy-D-xylulose-5-phosphate synthase (chloroplastic), the third marker, Affx_123243430 (R^2 = 23%), was located in the upstream of fatty acid 2-hydroxylase 1-like transcript variant X1, the fourth (Affx_123279219, R^2 = 25%) and fifth SNPs (Affx_123251060, R^2 = 16%) were located in the upstream of cytochrome P450 714A1-like and cytochrome P450 714A2-like, respectively. One SNP (Affx_123292815, R^2 = 23%) on chromosome two located in the upstream of auxin transporter-like protein transcript variant X1 was associated with β-carotene from the 2015 trial. On chromosome three, two SNPs including Affx_123282927 (R^2 = 77%), located in the downstream of cytochrome P450 93A3, and Affx_123264067 (R^2 = 26%), located in the upstream of cytochrome P450 93A3, showed highly significant association with β-cryptoxanthin in the 2016 trial at Limerick, SK (Figure 3.7 and Table 3.4 A). One marker, Affx_123251042 (R^2 = 26%), with a significant association to the mean of β-carotene concentration from the two-year trials was located in the downstream of cytochrome P450 93A3-like on chromosome four. Three additional SNPs on chromosome four showed strong association with β-cryptoxanthin concentration in 2016. The first marker, Affx_123240234 (R^2 = 76%), was found in the upstream of gibberellin 20 oxidase 2-like, the second marker, Affx_123255330 (R^2 = 22%), was located in the downstream of chlorophyllide, an oxygenase chloroplastic gene, and the third marker, Affx_123284451 (R^2 = 27%), was located upstream of NAD kinase chloroplastic-like. Markers Affx_123249382 (R^2 = 19%), Affx_123249312 (R^2 = 33%), Affx_123255905 (R^2 = 32%) and Affx_123291158 (R^2 = 33%), found on chromosome five close to abscisic acid receptor PYL4-like gene, showed strong association with the average of zeaxanthin concentration in two-year trials (Figure 3.7, Table 3.4 A). The rest 103 SNP markers that had a significant association with the concentration of various carotenoid components were in the vicinity of genes with unknown function in carotenoid biosynthesis (Table 3.4 B). One SNP

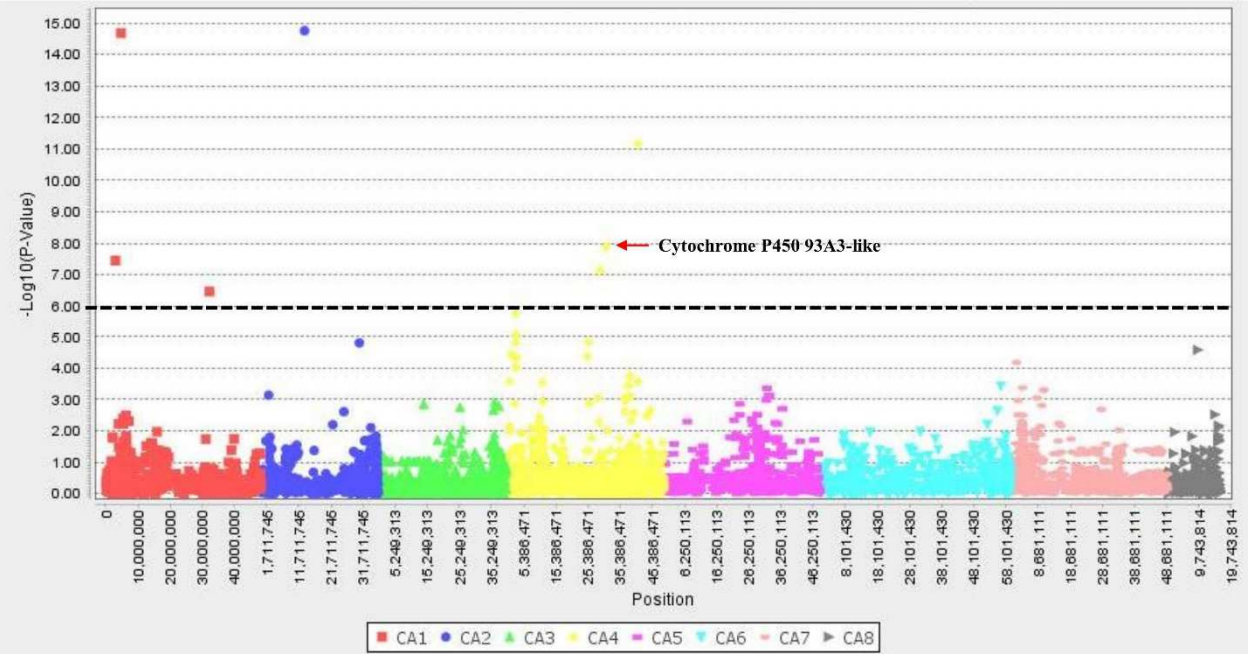
was significantly associated with lutein concentration. Moreover, 11 SNPs with violaxanthin, 25 SNPs with zeaxanthin, 16 SNPs with β-carotene and 50 SNPs with β-cryptoxanthin concentration were showing significant association in this study.



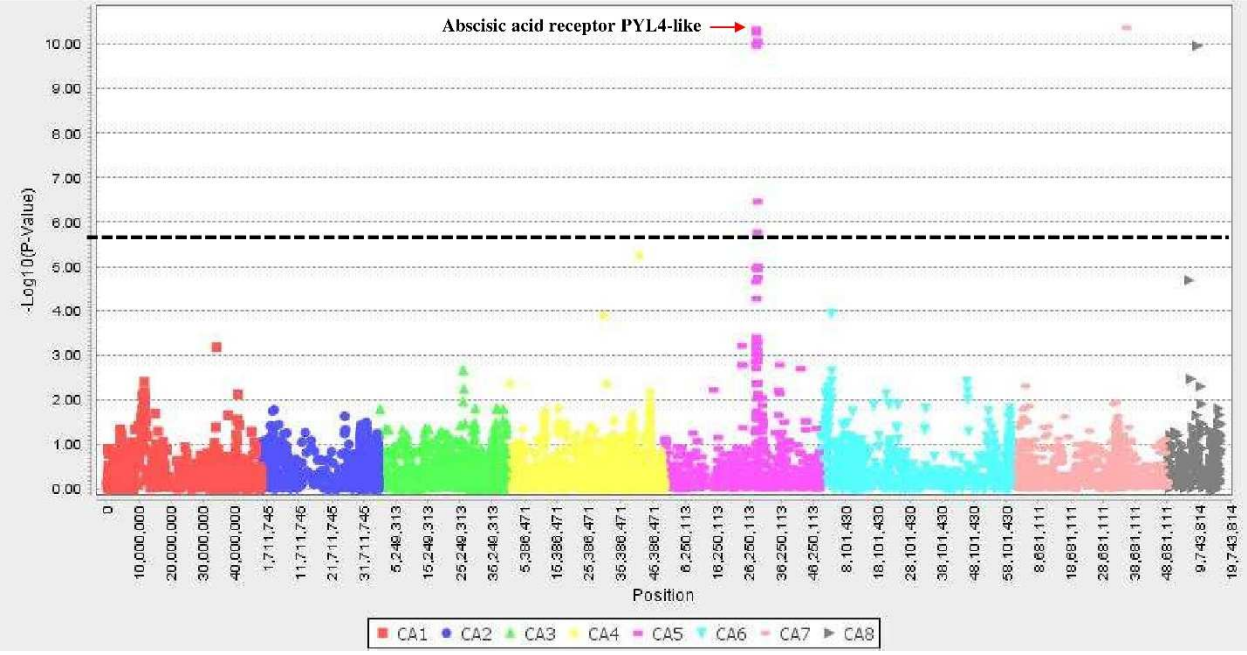
B: P-Values by Chromosome for β-Cryptoxanthin (2016)



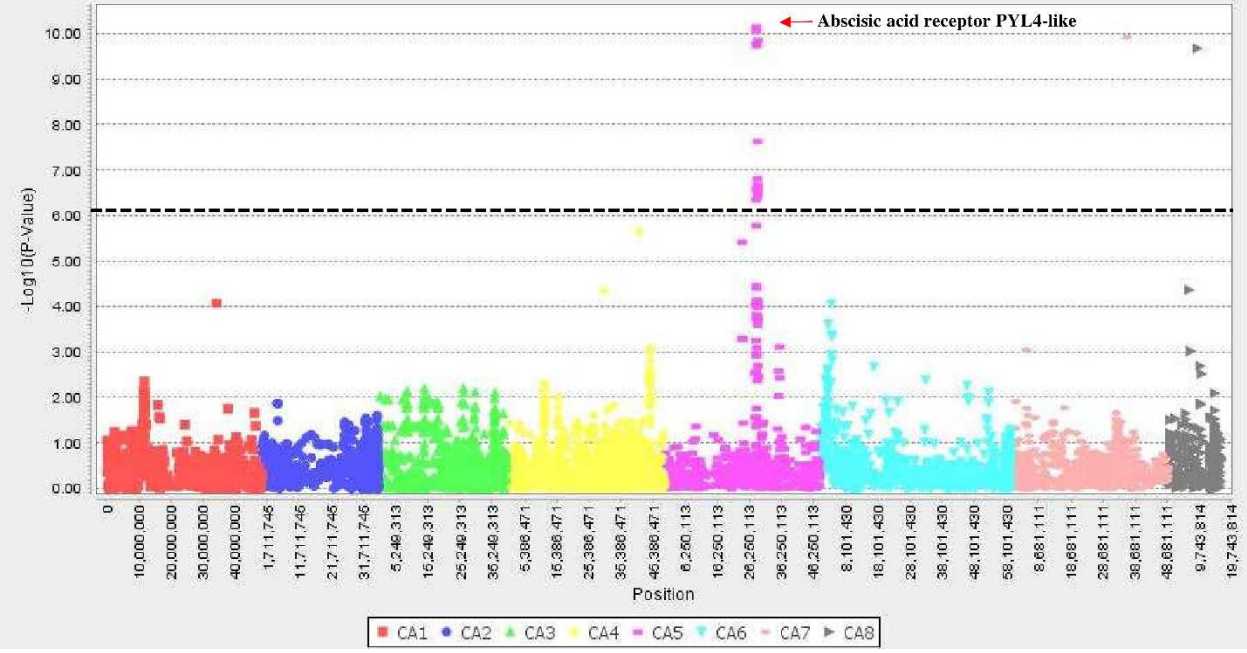
C: P-Values by Chromosome for β-Carotene (average of 2015-16)



D: P-Values by Chromosome for Zeaxanthin (2016)



E: P-Values by Chromosome for Zeaxanthin (average of 2015-16)



F: Quantile-quantile plot for carotenoid concentration (µg/g) in chickpea accessions

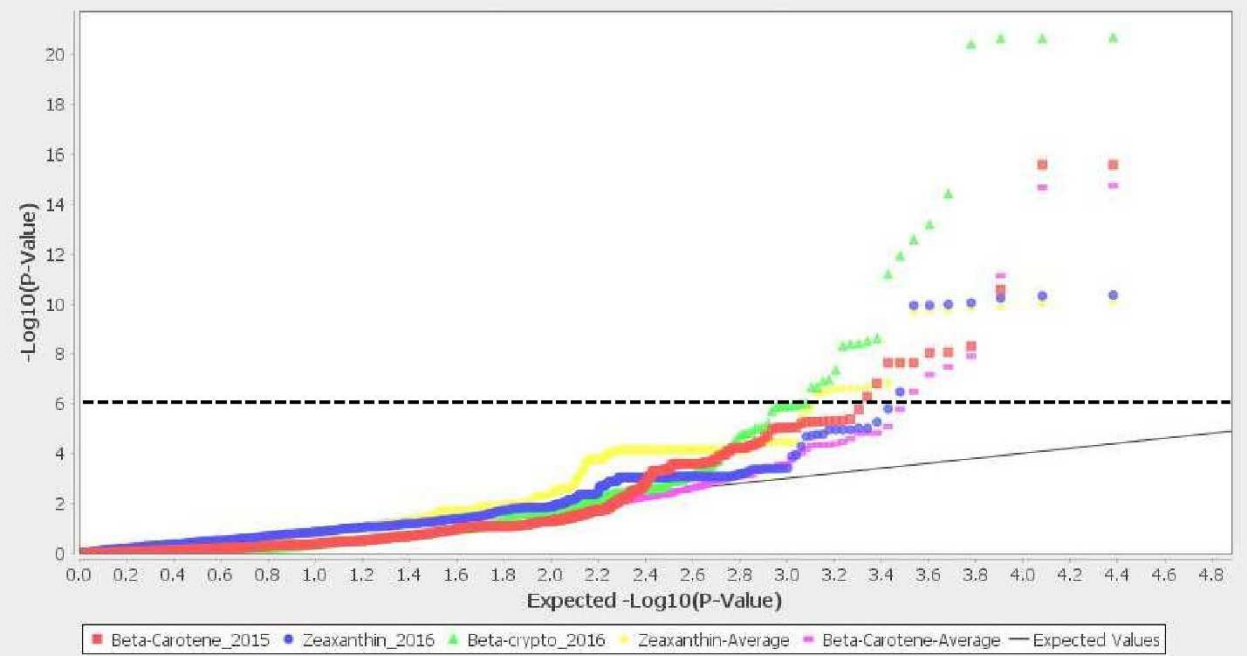


Figure 3.7. Manhattan plots for the concentrations of five carotenoids including violaxanthin, lutein, zeaxanthin, β-cryptoxanthin and β-carotene based on SNPs' P value (MAF ≥ 0.5) for Elrose 2015, Limerick 2016 and average of two years. Significant markers were found for β-carotene in 2015 (A), β-cryptoxanthin in 2016 (B), average of β-carotene in 2015 and 2016 (C), zeaxanthin in 2016 (D) and average of zeaxanthin in 2015 and 2016 (E). The plot F represent quantile-quantile (Q-Q) results from GWAS of β-carotene (2015), β-cryptoxanthin (2016), average of β-carotene in 2015 and 2016, zeaxanthin in (2016), and average of zeaxanthin in 2015 and 2016.

Table 3.4. Summary of association for different carotenoids including marker name, chromosome, physical position of the 17 SNP markers, P value and adjusted P value after Hochberg test, R^2 and associated genes with their function (A) and 103 SNP markers associated with genomic regions with unknown role in carotenoid biosynthesis (B).

A

Trait	Marker	Chrom	Position	P value	Adjusted P value (Hochberg test)	Marker R^2 (%)	Gene ID	Putative function
β-carotene (2015)	Affx_123277641	CA1	3028273	5×10^{-9}	8×10^{-5}	25	LOC101496685	Pyruvate decarboxylase 1
β-carotene (2015)	Affx_123269438	CA1	4713347	2×10^{-16}	4×10^{-12}	54	LOC101495911	1-deoxy-D-xylulose-5-phosphate synthase
β-carotene (2015)	Affx_123243430	CA1	7672148	2×10^{-8}	4×10^{-5}	23	LOC101504844	Fatty acid 2-hydroxylase 1, transcript variant X1
β-carotene (2015)	Affx_123279219	CA1	32245744	9×10^{-9}	1.5×10^{-5}	25	LOC101505060	Cytochrome P450 714A1
β-carotene (2015)	Affx_123251060	CA1	35230253	5.2×10^{-7}	8×10^{-4}	16	LOC101512027	Cytochrome P450 714A2
β-carotene (2015)	Affx_123292815	CA2	30436818	2.4×10^{-8}	4×10^{-4}	23	LOC101497816	Auxin transporter protein, transcript variant X1
β-cryptoxanthin (2016)	Affx_123282927	CA3	29778775	2.2×10^{-21}	3.7×10^{-17}	77	LOC101498247	Cytochrome P450 93A3
β-cryptoxanthin (2016)	Affx_123264067	CA3	29874406	3.3×10^{-9}	5.5×10^{-5}	26	LOC101498247	Cytochrome P450 93A3
β-cryptoxanthin (2016)	Affx_123247610	CA4	11086655	5.1×10^{-9}	8.4×10^{-5}	26	LOC101492973	Cytochrome P450 78A7
β-carotene (2015-16)	Affx_123251042	CA4	31002317	1.3×10^{-8}	2.1×10^{-4}	26	LOC101488523	Cytochrome P450 93A3
β-cryptoxanthin (2016)	Affx_123240234	CA4	36576040	3.8×10^{-21}	3.6×10^{-17}	76	LOC101504807	Gibberellin 20 oxidase 2-
β-cryptoxanthin (2016)	Affx_123255330	CA4	38283717	4.6×10^{-8}	7.5×10^{-4}	22	LOC101515232	Chlorophyllide a oxygenase chloroplastic
β-cryptoxanthin (2016)	Affx_123284451	CA4	38413220	4.4×10^{-9}	7.2×10^{-5}	27	LOC101492431	NAD kinase chloroplastic

Zeaxanthine (2015-16)	Affx_123249382	CA5	28335811	4.4×10^{-7}	7.3×10^{-3}	19	LOC101509736	Abscisic acid receptor PYL4
Zeaxanthin (2016)	Affx_123249312	CA5	28459661	4.6×10^{-11}	7.6×10^{-7}	33	LOC101509736	Abscisic acid receptor PYL4
Zeaxanthin (2016)	Affx_123255905	CA5	28539785	1×10^{-10}	1.6×10^{-6}	32	LOC101509736	Abscisic acid receptor PYL4
Zeaxanthin (2015-16)	Affx_123291158	CA5	28537227	5.6×10^{-11}	9.3×10^{-7}	33	LOC101509736	Abscisic acid receptor PYL4

B

Trait	Marker	Chromosome	Position	P value	Adjusted P value (Hochberg test)	Marker R^2 (%)
β-carotene (2015-2016)	Affx_123269438	CA1	4713347	2.08×10^{-15}	3.4×10^{-11}	50
β-cryptoxanthin (2016)	Affx_123282607	CA1	39814983	2.5×10^{-13}	4.2×10^{-9}	38
β-cryptoxanthin (2015-2016)	Affx_123282607	CA1	39814983	2.5×10^{-13}	4.2×10^{-9}	38
Violaxanthin (2015-2016)	Affx_123269438	CA1	4713347	1.4×10^{-12}	2.3×10^{-8}	38
Violaxanthin (2016)	Affx_123269438	CA1	4713347	2.4×10^{-12}	4×10^{-8}	37
β-carotene (2016)	Affx_123269438	CA1	4713347	3×10^{-10}	4.9×10^{-6}	30
Violaxanthin (2015)	Affx_123269438	CA1	4713347	1.8×10^{-9}	3×10^{-5}	27
β-carotene (2015)	Affx_123279219	CA1	32245744	9.6×10^{-9}	1.5×10^{-4}	25
β-carotene (2015)	Affx_123293125	CA1	3033294	2.4×10^{-8}	3.9×10^{-4}	24
β-carotene (2015-2016)	Affx_123277641	CA1	3028273	3.5×10^{-8}	5.9×10^{-4}	23
β-carotene (2015)	Affx_123247726	CA2	13519025	2.6×10^{-16}	4.3×10^{-12}	54
β-carotene (2015-2016)	Affx_123247726	CA2	13519025	1.7×10^{-15}	2.9×10^{-11}	50
Violaxanthin (2015-2016)	Affx_123247726	CA2	13519025	1.8×10^{-12}	3.09×10^{-8}	37
Violaxanthin (2016)	Affx_123247726	CA2	13519025	2.4×10^{-12}	4.03×10^{-8}	37
β-carotene (2016)	Affx_123247726	CA2	13519025	2.3×10^{-10}	3.8×10^{-6}	30

Violaxanthin (2015)	Affx_123247726	CA2	13519025	3.7×10^{-9}	6.2×10^{-5}	26
Lutein (2016)	Affx_123247726	CA2	13519025	2.7×10^{-6}	4.5×10^{-3}	15
β-cryptoxanthin (2016)	Affx_123294820	CA3	33973053	2.07×10^{-21}	3.4×10^{-17}	77
β-cryptoxanthin (2015-2016)	Affx_123294820	CA3	33973053	2.07×10^{-21}	3.4×10^{-17}	77
β-cryptoxanthin (2015-2016)	Affx_123282927	CA3	29778775	2.2×10^{-21}	3.7×10^{-17}	77
β-cryptoxanthin (2016)	Affx_123252817	CA3	35983077	2.2×10^{-21}	3.7×10^{-17}	77
β-cryptoxanthin (2015-2016)	Affx_123252817	CA3	35983077	2.2×10^{-21}	3.7×10^{-17}	77
β-cryptoxanthin (2015-2016)	Affx_123264067	CA3	29874406	3.3×10^{-9}	5.5×10^{-5}	26
β-cryptoxanthin (2016)	Affx_123276921	CA3	33798551	1.01×10^{-6}	1.6×10^{-3}	18
β-cryptoxanthin (2015-2016)	Affx_123276921	CA3	33798551	1.01×10^{-6}	1.6×10^{-3}	18
β-cryptoxanthin (2016)	Affx_123254892	CA3	33876202	1.3×10^{-6}	2.1×10^{-3}	17
β-cryptoxanthin (2015-2016)	Affx_123254892	CA3	33876202	1.3×10^{-6}	2.1×10^{-3}	17
β-cryptoxanthin (2016)	Affx_123258087	CA3	33734322	1.4×10^{-6}	2.4×10^{-3}	17
β-cryptoxanthin (2015-2016)	Affx_123258087	CA3	33734322	1.4×10^{-6}	2.4×10^{-3}	17
β-cryptoxanthin (2015-2016)	Affx_123240234	CA4	36576040	3.8×10^{-21}	6.3×10^{-17}	76
β-cryptoxanthin (2016)	Affx_123247260	CA4	30060330	3.6×10^{-15}	6.1×10^{-11}	49
β-cryptoxanthin (2015-2016)	Affx_123247260	CA4	30060330	3.6×10^{-15}	6.1×10^{-11}	49
β-cryptoxanthin (2016)	Affx_123262851	CA4	29031676	1.1×10^{-12}	1.8×10^{-8}	39
β-cryptoxanthin (2015-2016)	Affx_123262851	CA4	29031676	1.1×10^{-12}	1.8×10^{-8}	39
β-cryptoxanthin (2016)	Affx_123264482	CA4	30122488	6.2×10^{-12}	1.03×10^{-7}	36
β-cryptoxanthin (2015-2016)	Affx_123264482	CA4	30122488	6.2×10^{-12}	1.03×10^{-7}	36
β-carotene (2015-2016)	Affx_123285044	CA4	40934920	7.1×10^{-12}	1.1×10^{-7}	36
β-carotene (2015)	Affx_123250583	CA4	3277509	2.6×10^{-11}	4.2×10^{-7}	34
β-carotene (2016)	Affx_123285044	CA4	40934920	3.4×10^{-10}	5.7×10^{-6}	29
β-cryptoxanthin (2016)	Affx_123256279	CA4	44925182	2.5×10^{-9}	4.1×10^{-5}	27
β-cryptoxanthin (2015-2016)	Affx_123256279	CA4	44925182	2.5×10^{-9}	4.1×10^{-5}	27

β-cryptoxanthin (2016)	Affx_123283144	CA4	4141008	4.06×10^{-9}	6.6×10^{-5}	26
β-cryptoxanthin (2015-2016)	Affx_123283144	CA4	4141008	4.06×10^{-9}	6.6×10^{-5}	26
β-cryptoxanthin (2015-2016)	Affx_123284451	CA4	38413220	4.4×10^{-9}	7.2×10^{-5}	27
β-cryptoxanthin (2015-2016)	Affx_123247610	CA4	11086655	5.1×10^{-9}	8.4×10^{-5}	25
β-carotene (2015)	Affx_123266737	CA4	29118029	8.9×10^{-9}	1.4×10^{-5}	25
β-cryptoxanthin (2015-2016)	Affx_123255330	CA4	38283717	4.6×10^{-8}	7.5×10^{-5}	22
Violaxanthin (2015-2016)	Affx_123285044	CA4	40934920	5.1×10^{-8}	8.4×10^{-5}	22
β-carotene (2015-2016)	Affx_123266737	CA4	29118029	7.1×10^{-8}	1.1×10^{-3}	21
β-cryptoxanthin (2016)	Affx_123274064	CA4	24106288	1.3×10^{-7}	2.1×10^{-4}	18
β-cryptoxanthin (2015-2016)	Affx_123274064	CA4	24106288	1.3×10^{-7}	2.1×10^{-3}	18
β-carotene (2015)	Affx_123251042	CA4	31002317	1.5×10^{-7}	2.6×10^{-3}	20
Violaxanthin (2015-2016)	Affx_123251042	CA4	31002317	1.8×10^{-7}	2.9×10^{-3}	20
Violaxanthin (2016)	Affx_123285044	CA4	40934920	2.2×10^{-7}	3.7×10^{-3}	19
β-cryptoxanthin (2016)	Affx_123240719	CA4	24242732	2.2×10^{-7}	3.7×10^{-3}	17
β-cryptoxanthin (2015-2016)	Affx_123240719	CA4	24242732	2.2×10^{-7}	3.7×10^{-3}	17
β-cryptoxanthin (2016)	Affx_123276710	CA4	22369709	1.02×10^{-6}	3.7×10^{-3}	17
β-cryptoxanthin (2015-2016)	Affx_123276710	CA4	22369709	1.02×10^{-6}	1.06×10^{-3}	17
β-cryptoxanthin (2016)	Affx_123280311	CA4	24236706	1.19×10^{-6}	1.9×10^{-2}	17
β-cryptoxanthin (2015-2016)	Affx_123280311	CA4	24236706	1.19×10^{-6}	1.9×10^{-2}	17
Violaxanthin (2016)	Affx_123251042	CA4	31002317	1.2×10^{-6}	2×10^{-2}	17
β-cryptoxanthin (2016)	Affx_123274044	CA4	22309772	1.3×10^{-6}	2×10^{-2}	17
β-cryptoxanthin (2016)	Affx_123265090	CA4	22396749	1.3×10^{-6}	2×10^{-2}	17
β-cryptoxanthin (2016)	Affx_123250785	CA4	24103827	1.3×10^{-6}	2×10^{-2}	17
β-cryptoxanthin (2015-2016)	Affx_123274044	CA4	22309772	1.3×10^{-6}	2×10^{-2}	17
β-cryptoxanthin (2015-2016)	Affx_123265090	CA4	22396749	1.3×10^{-6}	2×10^{-2}	17
β-cryptoxanthin (2015-2016)	Affx_123250785	CA4	24103827	1.3×10^{-6}	2×10^{-2}	17

β-carotene (2015)	Affx_123248971	CA4	38472663	1.7×10^{-6}	2×10^{-2}	14
β-carotene (2015-2016)	Affx_123262539	CA4	2913155	1.7×10^{-6}	2×10^{-2}	15
Violaxanthin (2015)	Affx_123285044	CA4	40934920	2.06×10^{-6}	3.3×10^{-2}	16
β-cryptoxanthin (2016)	Affx_123301234	CA4	24166046	2.06×10^{-6}	3.3×10^{-2}	16
β-cryptoxanthin (2015-2016)	Affx_123301234	CA4	24166046	2.06×10^{-6}	3.3×10^{-2}	16
Zeaxanthin (2015-2016)	Affx_123266794	CA4	41183318	2.1×10^{-6}	3.5×10^{-2}	16
Zeaxanthin (2015-2016)	Affx_123249312	CA5	28459661	7.2×10^{-11}	1.1×10^{-6}	32
Zeaxanthin (2016)	Affx_123264199	CA5	28849221	8.5×10^{-11}	1.4×10^{-6}	32
Zeaxanthin (2015-2016)	Affx_123291158	CA5	28537227	8.7×10^{-11}	1.4×10^{-6}	32
Zeaxanthin (2016)	Affx_123288314	CA5	28559403	1.1×10^{-10}	1.8×10^{-6}	32
Zeaxanthin (2015-2016)	Affx_123264199	CA5	28849221	1.4×10^{-10}	2.3×10^{-6}	31
Zeaxanthin (2015-2016)	Affx_123255905	CA5	28539785	2.6×10^{-10}	2.7×10^{-6}	31
Zeaxanthin (2015-2016)	Affx_123288314	CA5	28559403	1.8×10^{-10}	3.3×10^{-6}	31
Zeaxanthin (2015-2016)	Affx_123269786	CA5	28801762	2.3×10^{-8}	3.9×10^{-4}	23
Zeaxanthin (2015-2016)	Affx_123272331	CA5	28725724	1.5×10^{-7}	2.5×10^{-3}	20
Zeaxanthin (2015-2016)	Affx_123273140	CA5	28809687	2.1×10^{-7}	3.5×10^{-3}	20
Zeaxanthin (2015-2016)	Affx_123253104	CA5	28852379	2.1×10^{-7}	3.5×10^{-3}	20
Zeaxanthin (2015-2016)	Affx_123256965	CA5	28602786	2.6×10^{-7}	4.3×10^{-3}	20
Zeaxanthin (2015-2016)	Affx_123247716	CA5	28665255	2.6×10^{-7}	4.3×10^{-3}	20
Zeaxanthin (2015-2016)	Affx_123284342	CA5	28731772	2.6×10^{-7}	4.3×10^{-3}	20
Zeaxanthin (2015-2016)	Affx_123243563	CA5	28842826	2.6×10^{-7}	4.3×10^{-3}	20
Zeaxanthin (2015-2016)	Affx_123243393	CA5	28915089	3.1×10^{-7}	5.2×10^{-3}	19
Zeaxanthin (2016)	Affx_123269786	CA5	28801762	3.4×10^{-7}	5.7×10^{-3}	19
Zeaxanthin (2015-2016)	Affx_123287286	CA5	28903558	3.6×10^{-7}	5.9×10^{-3}	19
Zeaxanthin (2015-2016)	Affx_123280355	CA5	28508712	1.6×10^{-6}	2.7×10^{-2}	17
Zeaxanthin (2016)	Affx_123272331	CA5	28725724	1.6×10^{-6}	2.7×10^{-2}	17

β-cryptoxanthin (2016)	Affx_123283993	CA7	28316739	6.4×10^{-14}	1.07×10^{-9}	44
β-cryptoxanthin (2015-2016)	Affx_123283993	CA7	28316739	6.4×10^{-14}	1.07×10^{-9}	44
Zeaxanthin (2016)	Affx_123280224	CA7	36110609	4.2×10^{-11}	4.05×10^{-7}	33
Zeaxanthin (2015-2016)	Affx_123280224	CA7	36110609	1.1×10^{-10}	1.9×10^{-6}	31
β-cryptoxanthin (2016)	Affx_123277895	CA7	5374060	1.1×10^{-7}	1.9×10^{-4}	21
β-cryptoxanthin (2015-2016)	Affx_123277895	CA7	5374060	1.1×10^{-7}	1.9×10^{-4}	21
β-cryptoxanthin (2016)	Affx_123271798	CA7	23860944	2.2×10^{-7}	3.7×10^{-4}	17
β-cryptoxanthin (2015-2016)	Affx_123271798	CA7	23860944	2.2×10^{-7}	3.7×10^{-4}	17
Zeaxanthin (2016)	Affx_123297553	CA8	9196236	3.09×10^{-10}	1.8×10^{-6}	31
Zeaxanthin (2015-2016)	Affx_123297553	CA8	9196236	2.1×10^{-10}	3.5×10^{-6}	30

3.3.7. Pearson correlation for carotenoids and seed compositions

Concentration of total essential amino acids, including phenylalanine, valine, leucine, isoleucine, lysine, threonine, tryptophan, methionine, and histidine showed a negative correlation with violaxanthin (r= -0.16, P<0.05). Total protein concentration was negatively correlated with violaxanthin (r= -0.15, P= P<0.05), but positively correlated with total essential amino acids (r=0.76, P<0.001), respectively. Total fat showed a negative correlation with violaxanthin (r= -0.19, P<0.01) and lutein (r= -0.17, P= P<0.05; Table 3.5).

Table 3.5. Pairwise Pearson correlation coefficients for carotenoid components including violaxanthin, lutein, zeaxanthin, β-carotene and total carotenoid concentration (µg g^{-1}) as well as total essential amino acids and fat across 172 chickpea accessions grown in Elrose in 2015.

	2	3	4	5	6	7	8 (Total fat)
Violaxanthin (1)	0.35^{***}	0.11^{ns}	0.49^{***}	0.41^{***}	-0.16^{*}	-0.15^{*}	-0.19^{**}
Lutein (2)		0.80^{***}	0.36^{***}	0.99^{***}	0.13^{ns}	0.04^{ns}	-0.17^{*}
Zeaxanthin (3)			0.07^{ns}	0.83^{***}	0.15^{*}	0.03^{ns}	0.14^{ns}
β-carotene (4)				0.39^{***}	-0.07^{ns}	0.04^{ns}	-0.19^{ns}
Total carotenoids (5)					0.12^{ns}	0.03^{ns}	-0.14^{ns}
Amino acid (6)						0.76^{***}	0.12^{ns}
Total protein (7)							-0.01^{ns}

Note: *, **, *** are significant at P ≤ 0.05, 0.01 and 0.001, respectively; ns, non-significant.

3.4. Discussion

The current GWAS allowed the identification of SNP markers significantly associated with concentrations of various carotenoid components in chickpea seeds, which potentially can be used to assist in breeding for improvement of provitamin A level. Previous studies demonstrated that a small number of loci with moderate to large effects are responsible for carotenoid content in maize (Wong et al; 2004; Chander et al., 2008; Kandianis et al., 2013). The chickpea accessions used in the current analysis were a combination of Canadian breeding lines and international accessions that represents both local and global variation (Table A1). Both PCA and structure analysis revealed that the genotypic panel consisted of two major groups; group I with the majority of kabuli type and group II with the majority of desi type. There are some admixtures of kabuli and desi backgrounds which might be explained by the origination of kabuli from desi type (Moreno and Cubero, 1978). The level of LD between a marker and a QTL determines the power of a

population for the association study (Xu et al., 2017b). Determining the rate of LD decay is important in association analysis as it gives an idea of the minimum number of markers needed for the analysis (Diapari et al., 2014; Thudi et al., 2014). Several factors including mating pattern, selection, mutation, migration and genetic drift affect LD decay in a population (Flint-Garcia et al., 2003). Higher marker density is required for the case of rapid LD decay and vice versa (Xu et al., 2017b). Self-pollinating crops like chickpea have lower LD decay compared to maize (cross-pollination) with the LD decay at a short distance (0.01-0.1 Mb; Lu et al., 2011; Diapari et al., 2014). The high level of homozygosity in self-pollinating plants makes the recombination events less effective on LD decay (Vos et al., 2017).

In the current study, the LD started to decay at 0.6 Mb which is smaller than the value (1-2 Mb) previously reported in chickpea (Diapari et al., 2014). The difference may be due to the greater number of accessions and the method of genotyping used in this study. Chickpea population with high genetic diversity showed lower LD decline (0.4-0.6 Mb) compared to the genetically less diverse population (0.7 to 0.8 Mb; Saxena et al., 2014b; Kujur et al., 2015). The estimates of LD decays in later two studies were close to the value obtained in our study. Also, the LD decay obtained in the current study is comparable to that reported in rice, another self-pollinating crop, with the LD decay of 0.37 Mb (Reig-Valiente et al., 2016). Based on the LD decay of 0.6 Mb obtained in the current genotypic panel, theoretically 1,233 markers would be sufficient for an association analysis. The current study used 24,095 SNP makers with relatively uniform distribution across the chickpea genome to increase the chance of capturing genomic regions associated with concentration of individual carotenoids.

The total number of SNPs associated with different carotenoid components as well as total carotenoid concentration after FDR test were 120 markers. Seventeen markers were in the vicinity to the genes with known function in carotenogenesis, while 103 markers were in the genomic regions with the unknown contribution to carotenoid concentration. Only one SNP associated with lutein was found among the 103 markers and none was associated with total carotenoids. New finding, the ‘‘omnigenic’’ model, suggests that association signal for the complex trait can be distributed in the genome in the vicinity to the genes without direct contribution to the phenotype (Boyle et al., 2017). Due to structural variations such as insertions, deletions, copy number variations, translocations, and transversions, the SNP genotyping does not always cover all variation in the genome associated with specific traits (Saxena et al., 2014a; Wang et al., 2014b;

Thudi et al., 2016). The current study revealed many SNPs that are not located within the known carotenoid genes. Most of these SNPs are located in the intergenic or intragenic regions, which are not flanked by the gene(s) of interest (De la Cruz et al., 2010); as such a sliding window-based approach (Petersen et al., 2013) was applied in this study.

The SNP marker Affx_123269438 which is in LD with the1-deoxy-D-xylulose-5-phosphate synthase (*DXS*) showed highly significant association ($P = 4 \times 10^{-12}$) with β-carotene concentration in the genotypic panel. The *DXS* is one of the rate-limiting enzymes in the MEP pathway and its overexpression positively affects isoprenoid synthesis (Estévez et al., 2001; Zhao et al., 2011). Engineering of *DXS* in *Escherichia coli* resulted in higher β-carotene production (Yang and Guo, 2014). The key role of *DXS* in synthesis and accumulation of carotenoids has been reported in several plant species including tomato (Enfissi et al., 2005), *Arabidopsis thaliana* (Estévez et al., 2001), maize (Vallabhaneni and Wurtzel, 2009) and soybean (Zhang et al., 2009a). As such, marker Affx_123269438 can be considered a potential marker for use in selection in the breeding program. Marker Affx_123277641 located in the upstream of pyruvate decarboxylase 1 (*PDC*) gene also showed significant association ($P = 8 \times 10^{-5}$) with β-carotene in chickpea. The sequence, substrate target and catalytic activity, of *DXS* are similar to *PDC* and transketolases and all categorized as thiamine diphosphate (TPP)-dependent (Sprenger et al., 1997; Lange et al., 1998). Synthesis of 1-deoxy-D-xylulose 5-phosphate (DXP) in the primary step of the MEP pathway is performed by pyruvate decarboxylation. This reaction is under the control of transketolases or the E1 subunit of *PDC* or pyruvate dehydrogenase (Rodriguez-Concepcion, 2002). In the case of orange melon (*Cucumis melo*), high expression level of the genes in the MEP pathway was positively correlated with β-carotene synthesis (Yuan et al., 2015). Six SNPs were located in the proximity of different cytochrome P450 (P450s) gene family with strong association with β-cryptoxanthin and β-carotene (Table 3.4 A). The P450 members belong to a large family of metabolic enzymes that control the production of lignin precursors and are involved in hormone homeostasis (Nelson and Werck-Reichhart, 2011).

Cytochrome P450 714A1 (*CYP714A1*) and Cytochrome P450 714A2 (*CYP714A2*) are catalysis gibberellic acid (GA) metabolism (Hamberger and Bak, 2013). In *Arabidopsis thaliana, CYP714A2* synthesized various GA products with oxidations of C and D rings in the ent-kaurene scaffold (Wang et al., 2016). Marker Affx_123240234 which is located near gibberellin 20 oxidase-2 gene showed a highly significant association ($P= 3.6 \times 10^{-17}$) with β-cryptoxanthin

concentration. In the last step of GA biosynthesis, gibberellin 20 oxidase (*GA20ox*) converts GA12 and GA53 to GA9 and GA20, respectively (Rieu et al., 2008). Biosynthesis of GA is dependent on geranylgeranyl diphosphate (GGPP), the main precursor of isoprenoid molecules including carotenoids (Giuliano, 2017), as such GA production partly regulates carotenoid accumulation in plants (Tuan et al., 2013). Strigolactone is produced by two cleavage enzymes *CCD7* and *CCD8* and is involved in carotenoid regulation (Lauressergues et al., 2015).

Four SNP markers, i.e., Affx_123249382, Affx_123249312, Affx_123255905, and Affx_123291158 were located close to *PYL4*, an abscisic acid receptor, with a significant association with zeaxanthin concentration in the current chickpea panel (Table 3.4 A). *PYL4* belongs to a superfamily of protein receptors (Ma et al., 2009). Xanthoxin, the precursor of abscisic acid (ABA), is produced in the last step of the carotenoid pathway through the activity of 9-cis-epoxycarotenoid dioxygenase (*NCED*) on 9-cis isomers of epoxycarotenoids (Schwartz, 1997). The signaling pathway over abiotic stress is regulated by ABA in plants (Tuteja, 2007). Thus, abiotic stresses and ABA synthesis can affect the accumulation of carotenoids (Cazzonelli and Pogson, 2010; Giuliano, 2017). Carotenoid biosynthesis is positively correlated with ABA concentration in Arabidopsis seeds (Lindgren, 2003) in which the reduction of the content of xanthophylls, like lutein and zeaxanthin, decreased the ABA concentration in the plant (Ren et al., 2007b). β-carotene, lutein and zeaxanthin stimulated the activity of 3-indolilacetic acid (IAA) and salicylic acid (SA) in *Wolffia arrhizal* under photoautotrophic conditions (Czerpak et al., 2002). Marker Affx_123292815, linked to an auxin transporter, transcript variant X1, was associated with β-carotene concentration in this study. Arabidopsis with mutant ζ-carotene desaturase (*ZDS*) affected auxin responses during plant development (Avendano-Vazquez et al., 2014). The balance of auxin-ethylene regulates the accumulation of carotenoids over the ripening period in tomato. For instance, auxin induces the key genes for β-xanthophyll and lycopene synthesis (Su et al., 2015). One means for auxin transport in plants is the cell-to-cell system or auxin transporting protein that facilitates the mobilization of the hormones in response to various developmental changes (Zazímalová et al., 2010). Chlorophyllide A oxygenase (*CAO*) is the key enzyme for biosynthesis of Chl b in plants (Tomitani, 1999). In the current GWAS we found that Affx_123255330 marker close to *CAO* showed a significant association with β-cryptoxanthin in chickpea. Overexpression of *CAO* in *Arabidopsis* resulted in the reduction of β-carotene level (Tanaka and Tanaka, 2005). However, the relationship between *CAO* expression and various

carotenoids needs further investigation. Fatty acids and carotenoids are similar in terms of structure and solubility (Shanklin and Cahoon, 1998). Significant variation for carotenoids was observed in the current chickpea accessions which corresponds to cotyledon colour diversity (Ashokkumar et al., 2014, 2015; Rezaei et al., 2016). Cotyledon colour is a monogenic trait that was mapped to chromosome 8. The green cotyledon chickpea cultivar CDC Jade had the highest level of carotenoid concentration among the accessions. Lower hydroxylation and cleavage activity due to the stay-green gene (*SGR*) mutation elevated the carotenoid level in chickpea cultivars with green cotyledons (Ashokkumar et al., 2015; Rezaei et al., 2016). On average the desi accessions had higher carotenoid concentration than kabuli type due to higher pigmentation in their cotyledons.

The effect of environment was significant on violaxanthin and zeaxanthin concentrations, and the effect of year was significant on β-carotene concentration (Ashokkumar et al., 2014). However, in the current study the effects of environment, genotype, and genotype by environment interaction were significant on the concentration of all carotenoids which could be due to a larger population and high contrast in environments. Moderate stress may positively increase the carotenoid concentration as carotenoids are involved in antioxidant activity and dissipation of excess energy (García-Plazaola et al., 2012; Fanciullino et al., 2014). Up-regulation of *PSY3* under salt stress was observed in rice (Welsch et al., 2008). Overall, carotenoid concentration across the accessions was greater in 2016 than in 2015, which may have been due to warmer/drier conditions at Limerick in 2016.

The concentration of most carotenoids was negatively correlated with seed weight. The results are in agreement with the findings from the previous study by Ashokkumar et al. (2015). Chromosomal linkage and pleiotropy could be responsible for the negative correlation between seed weight and lutein concentration in chickpea (Abbo et al., 2005; Ashokkumar et al., 2015). Molecular markers tightly linked to lutein concentration will be helpful to break this linkage by selecting progenies with large seed size and high lutein level via marker-assisted selection. Negative correlation between β-carotene and maturity was reported by Rezaei et al. (2016). In soybean, β-carotene was positively correlated with chlorophylls and green seed, and its level decreased by maturity period (Monma et al., 1994). Up to 70% of provitamin A carotenoids in maize can be degraded during 4 to 6 months storage (Owens et al., 2014), as such to achieve satisfactory level of carotenoids, higher carotenoid content in seeds with focus on breeding for green chickpeas is needed. The SNPs with significant association with the concentrations of

various carotenoids obtained in this study can be used to assist selection in breeding programs to improve provitamin A levels in chickpea.

In conclusion, genomic regions associated with carotenoid concentration in chickpea accessions were identified using a combination of high-throughput genotyping and high precision phenotyping using HPLC. Carotenoids as quantitative traits are under the control of moderate number of genes. The concentration of carotenoids and their biosynthesis are affected by environments. The high number and well distributed SNPs across the chickpea genome allowed the identification of the most of important SNP loci associated with carotenoids. Candidate genes involved in the biosynthesis of apo-carotenoids and precursor of carotenoids had the largest effect on carotenoid concentration. The SNPs strongly associated with various carotenoids can be used to aid selection for developing chickpea cultivars with boosted provitamin A levels in seeds

CHAPTER 4. QTL MAPPING FOR CAROTENOID CONCENTRATION AND ITS RELATIONSHIP WITH COTYLEDON COLOUR IN THREE CHICKPEA POPULATIONS

Preface

The following chapter focused on objectives three and four on my Ph.D. project, which was an attempt to identify quantitative trait loci (QTL) associated with different carotenoid components and to examine the relationship between cotyledon colour and carotenoid concentration in chickpea. For these purposes, three chickpea populations were developed based on differences in cotyledon colour and carotenoid concentration. The populations were genotyped using the 50K Axiom® *CicerSNP* Array and analyzed for their carotenoids using HPLC. Significant positive relationship between cotyledon colour and carotenoid concentration was found in this study. Five to eight QTLs for different carotenoid components were found in three populations and cotyledon colour as a monogenic trait was successfully mapped on the same linkage group in all populations.

4.1. Introduction

Chickpea (*Cicer arietinum* L., 2n = 16) with relatively small genome size (740 Mb; Arumuganathan and Earle, 1991) is the second most important pulse crop globally in term of production. The combination of protein, carbohydrate, and vitamins, especially provitamin A carotenoid in chickpea seeds, makes chickpea an important diet in many developing countries (Abbo et al., 2005; Abu-Salem and Abou-Arab, 2011). Chickpeas with green or orange cotyledon colour contain high levels of provitamin A such as β-carotene and β-cryptoxanthin (Ashokkumar et al., 2014; Rezaei et al., 2016). The crop is an ideal model for carotenogenesis in legumes due to the availability of its genome sequence (Varshney et al., 2013a) and the existence of substantial cotyledon colour variations. The expression levels of some genes from the carotenoid biosynthesis pathway have been analyzed in chickpea by Rezaei et al. (2016); however, the genes that are responsible for cotyledon colour and those involved in isoprenoid pathway have not been studied.

The relationship between fruit or root colour and genes in the carotenoid pathway was reported in tomato (Liu et al., 2003) and carrot (Just et al., 2009). One approach to identify the genes associated with carotenoid components is linkage analysis using high resolution map (Roorkiwal et al., 2017) and quantitative trait loci (QTL) mapping (Abbo et al., 2005). The availability of a saturated genetic map can increase the power and accuracy in detecting QTLs (Varshney, 2016). Recently, two chickpea recombinant inbred line (RIL) populations were genotyped using 50 K Axiom® *CicerSNP* chip and dense genetic maps were constructed by Roorkiwal et al. (2017). Mapping QTLs associated with important carotenoids has been reported in different crops. In a cross between orange and white carrot, two loci, *Y* and Y_2, 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 root (Just et al., 2009; Ellison et al., 2017). QTL analysis successfully identified important QTLs for carotenoid components as well as yellow pigment concentration and yellow colour index in durum wheat (Blanco et al., 2011). Also, relatively stable QTLs for carotenoids and flour colour were found using a set of recombinant inbred lines in wheat (Zhao et al., 2013). In cassava, five and three QTLs were responsible for provitamin A content and root colour, respectively (Ana et al., 2013). QTLs with strong effect on potato flesh pigmentation were identified on chromosome 5, 8 and 9. Additionally, a transcript regulator for anthocyanin biosynthesis in potato was co-localized with the QTL on chromosome 9 (Zhang et al., 2009b). Candidate genes for carotenoid metabolism, including lycopene epsilon cyclase, carotenoid cleavage dioxygenase1, and β-carotene hydroxylase, were mapped using maize $F_{2:3}$ population (Kandianis et al., 2013). The QTLs identified in the RIL population of maize accounted for 4-47% variations for carotenoids (Jittham et al., 2017). QTLs for provitamin A (β-carotene) and lutein content were previously reported in chickpea (Abbo et al., 2005).

The main objective of this study was to map the QTLs associated with concentration of major carotenoids in three chickpea populations derived from crossing parental lines with different cotyledon colours. The study also evaluated the relationship between cotyledon colours and carotenoid levels in each population.

4.2. Materials and methodology

4.2.1. Plant materials

Five F_2 populations were initially developed in greenhouse condition at the Crop Development Center (CDC), the University of Saskatchewan in 2014 (Table 4.1). The F_2 seeds were planted in 2 L pots filled with Sunshine mix #4 media (SunGrow, Seba Beach, Alberta, Canada) at the College of Agriculture and Bioresources greenhouse in July 2014. The NPK fertilizer (20-20-20) was added to each pot (3 g L^{-1}) three times after plants reached the height of 20 cm. The watering was done manually two to three times a week depending on the growth stage and it stopped by the time plants turned yellow. Individual plants were harvested with hands and the pods were threshed manually. Chickpea cultivars were selected based on cotyledon colour variation as well as carotenoid level differences. CDC Jade and CDC Frontier were showing highest and lowest carotenoid concentration among the selected cultivars respectively. CDC Verano and CDC Jade were the green cotyledon chickpeas and rest were yellow for cotyledon colour. In each population, one green cotyledon chickpea was in a cross with a yellow cultivar. Other criteria like early maturity, resistance to Ascochyta blight and seed weight were considered for population development. CDC Frontier was selected for it's highest 1000 seed weight among the cultivars. Also, CDC Jade for early maturity and ICC4475 for high resistance to Ascochyta blight disease were chosen for creating the biparental populations.

4.2.2. Inheritance of cotyledon colour

One hundred F_2 seeds from each population were selected for cotyledon colour scoring. Each seed was cut in half using a plier and the cotyledon colour was assessed visually. Goodness of fit of the observed to the expected segregation ratios (3 yellow: 1 green) of cotyledon colour in each of the five F_2 populations was performed using the Chi-square test.

Table 4.1. Five F_2 populations derived from biparental crosses of six chickpea cultivars including kabuli (CDC Verano and CDC Frontier and CDC 441-34) and desi types (CDC Jade, CDC Cory and ICC4475) with different cotyledon and seed coat colours. The number of individuals in each F_2 population and the harvested F_3 seeds from individual F_2 plant are indicated.

Parent 1	Parent 2	F_2 population size	Average no. of $F_{2:3}$ seeds
CDC Verano (green cotyledon and transparent seed coat)	CDC 441-34 (yellow cotyledon and off-white seed coat)	120	35
CDC Verano	CDC Frontier (yellow cotyledon and transparent seed coat)	120	35
CDC Jade (green cotyledon and green seed coat)	CDC Frontier	118	35
CDC Cory (yellow cotyledon and brown seed coat)	CDC Jade	120	35
ICC4475 (yellow cotyledon and black seed coat)	CDC Jade	118	35

4.2.3. Carotenoid measurement

The carotenoid measurement was done on the selected three $F_{2:3}$ populations. Four randomly selected F_3 seeds from each F_2 plant of CDC Jade × CDC Frontier, CDC Cory × CDC Jade and ICC4475 × CDC Jade populations (Table 4.1), were used for HPLC assay. The concentration of five carotenoid components including violaxanthin, lutein, zeaxanthin, β-cryptoxanthin, and β-carotene were analyzed. Carotenoid extraction and HPLC procedure were performed based on the method described by Rezaei et al. (2016).

4.2.4. Statistical analysis

Test of the goodness of fit of cotyledon colour segregation and mean comparison for carotenoid concentration in green, segregating and yellow groups within each population were done using SAS 9.3 (SAS Institute Inc., Cary, NC).

4.2.5. DNA extraction and genotyping

The total DNA from leaves of individual F_2 plants from the three selected populations including CDC Jade x CDC Frontier, CDC Cory x CDC Jade, and ICC4475 x CDC Jade was extracted using the method as described in chapter 3. After checking the quality and quantity of the DNA, the samples were sent to ICRICAT, Hyderabad, Patancheru, India for genotyping using the 50 K Axiom® *CicerSNP* chip (Roorkiwal et al., 2017).

4.2.6. Map construction and QTL analysis

Each SNP locus was tested against the expected segregation ratio of 1:2:1 in an F_2 population using the Chi-square test in MapDisto 2.0 software (Heffelfinger et al., 2017). The SNPs with a 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 colour segregation is considered as a major gene and it is integrated directly into the genetic map along with the SNP markers. In the case of CDC Jade × CDC Frontier, CDC Jade with green cotyledon colour was used as female and CDC Frontier with yellow cotyledons as male; progenies with green, yellow, and segregating for cotyledon colours were converted to A, B and, H, respectively. The opposite was done for CDC Cory × CDC Jade and ICC4475 × CDC Jade populations since CDC Jade was used as male and the yellow cotyledon cultivars as female parent. The QTL analysis was done using QTL IciMapping V3.3 software (Meng et al., 2015). The inclusive composite interval mapping (ICIM) approach based on bin markers was used for mapping QTLs through ICIM-ADD option in the software. One thousand permutations were done to set the LOD threshold with type I error of ≤0.05.

4.3. Results

4.3.1. Inheritance study

Chi-square analysis showed that cotyledon colour is under the control of a single gene with yellow cotyledon is dominant over green cotyledon (Table 4.2).

Table 4.2. Chi-square test for the goodness of fit of the observed to the expected segregation ratios (3:1, yellow vs. green) of cotyledon colour in five F_2 populations derived from crosses of cultivars with contrasting cotyledon colour. *df* =1, $\chi^2(0.05) = 3.841$, $\chi^2(0.01) = 6.635$.

Parent 1	Parent 2	Yellow cotyledon	Green cotyledon	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

Note: CDC Frontier, yellow cotyledon kabuli; CDC Verano, green cotyledon kabuli; CDC 441-34, yellow cotyledon kabuli; CDC Jade, green cotyledon desi; CDC Cory, yellow cotyledon desi; ICC4475, black seed coat yellow cotyledon desi.

4.3.2. Carotenoid measurement

The seeds of each $F_{2:3}$ family from each population were divided into three groups based on cotyledon colours including yellow, green, and segregating. In CDC Jade × CDC Frontier population, 40 families were yellow, 20 were green, and 58 were segregating. In the cross between CDC Cory × CDC Jade, 37 families were yellow, 19 were green, and 64 were segregating. The families from the cross between ICC4475 and CDC Jade were divided into 42 yellow, 21 green, and 55 segregating for cotyledon colour (Figure 4.1).

A considerable variation for the concentration of different type of carotenoids was observed in all populations. Individuals with green cotyledon were showing almost the highest concentration of carotenoid components in three chickpea populations. There were genotypes in each population with zero level of β-carotene and β-cryptoxanthin. These components were detectable in individuals with green or orange cotyledon colour. Also, the zero level of violaxanthin only observed in population CDC Jade x CDC Frontier. The epistatic effect was calculated in all populations (data not shown) which confirmed the transgressive segregate effect on different carotenoid components in chickpea populations. For example, total carotenoid concentration in some individuals from populations CDC Cory x CDC Jade and ICC4475 x CDC Jade was two times more than the best parent (CDC Jade). In the first population (CDC Jade x CDC Frontier) some genotypes were showing 50% more total carotenoid level than CDC Jade.

The mean comparison of total carotenoid concentration in three groups including green, segregating and yellow cotyledon colour within each biparental population showed that green category was significantly different from the yellow group and segregating individuals were placed between the green and yellow group in terms of carotenoid level in population CDC Jade x CDC Frontier. In the second population CDC Cory x CDC Jade, the green was in between segregating and yellow progenies. In population ICC4475 x CDC Jade both green and segregating plants were in the same group with significant differences from yellow genotypes (Table 4.3). The positive effect of green cotyledon colour on total carotenoid concentration was clearly observed in three F_2 populations in this study. The results of carotenoid measurements using HPLC including violaxanthin, lutein, zeaxanthin, β-carotene, and β-cryptoxanthin from the three populations are presented in Table A4, 5, and 6 and the distribution of each component is shown in Figure 4.2

Table 4.3. Mean comparison of total carotenoid concentration ($\mu g\ g^{-1}$) ± Se measured by HPLC in three F_2 populations including CDC Jade x CDC Frontier, CDC Cory x CDC Jade and ICC4475 x CDC Jade using Fisher's least significant difference (LSD) test at significant level of 5%.

Population	Cotyledon colour	Mean for total carotenoids ($\mu g\ g^{-1}$)	Group
CDC Jade x CDC Frontier	Green	37.04 ± 1.78	A
CDC Jade x CDC Frontier	Segregating	34.22 ± 1.05	AB
CDC Jade x CDC Frontier	Yellow	30.83 ± 1.26	B
CDC Cory x CDC Jade	Segregating	55.01 ± 1.39	A
CDC Cory x CDC Jade	Green	49.28 ± 2.56	AB
CDC Cory x CDC Jade	Yellow	41.79 ± 1.83	B
ICC4475 x CDC Jade	Segregating	54.42 ± 1.21	A
ICC4475 x CDC Jade	Green	51.18 ± 1.94	A
ICC4475 x CDC Jade	Yellow	44.42 ± 1.4	B

Note: mean groups with the same letter are not significantly different based on LSD at 5%.

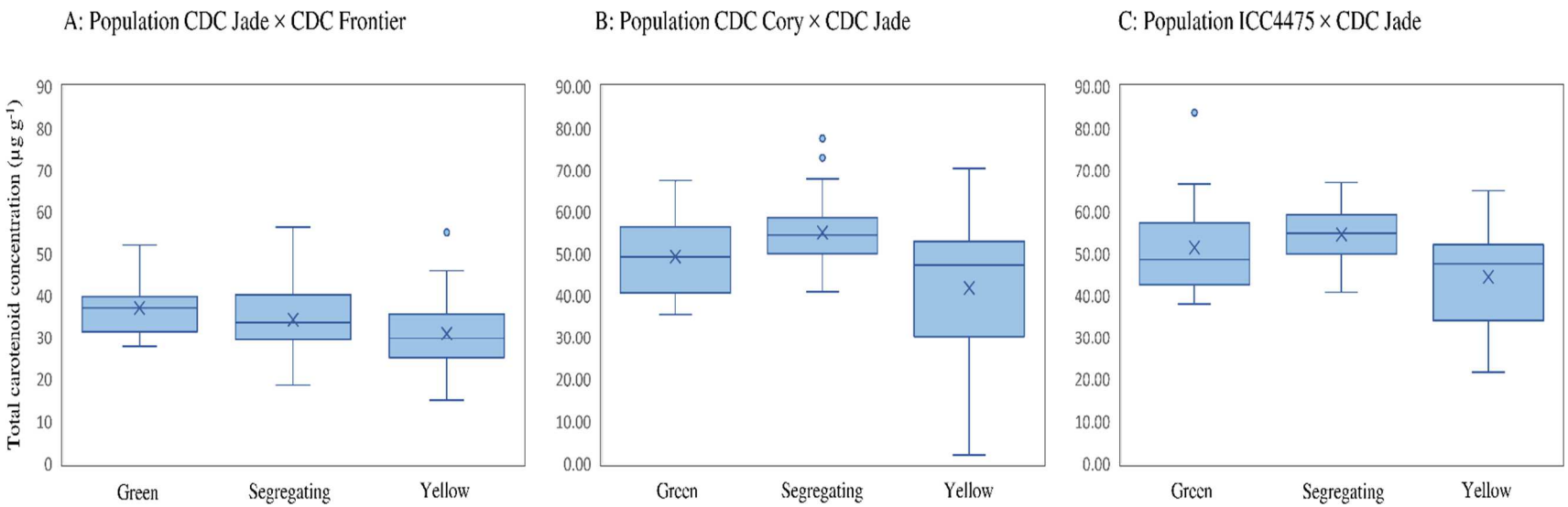
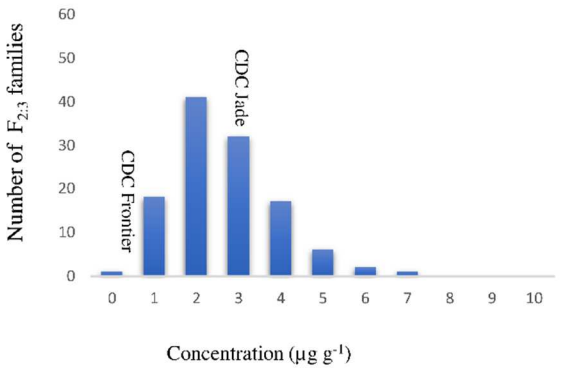
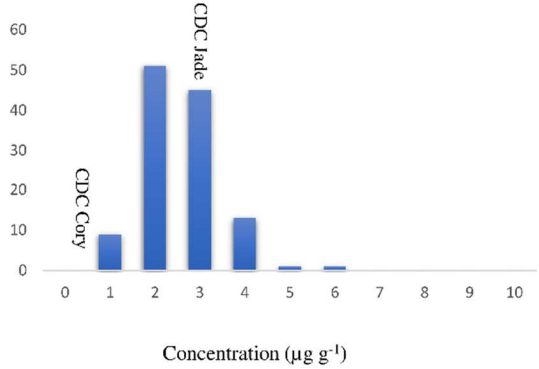


Figure 4.1. Box plots of total carotenoid concentration (µg g^{-1}) measured based on HPLC method for each family with different cotyledon colour in $F_{2:3}$ seeds of three chickpea populations including CDC Jade × CDC Frontier (A), CDC Cory × CDC Jade (B) and ICC4475 × CDC Jade (C).

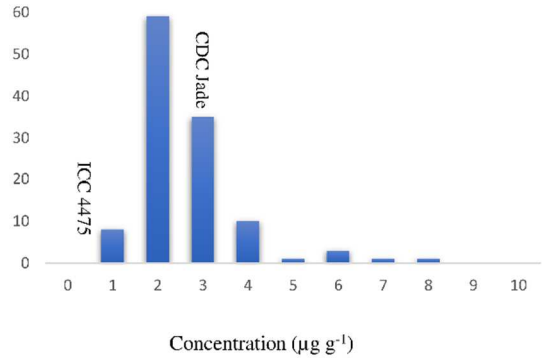
A1: Distribution of violaxanthin in population JF



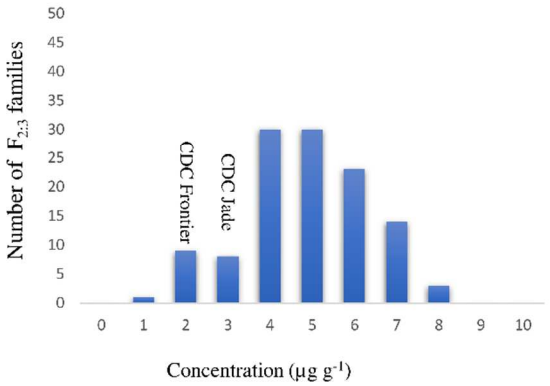
A2: Distribution of violaxanthin in population CJ



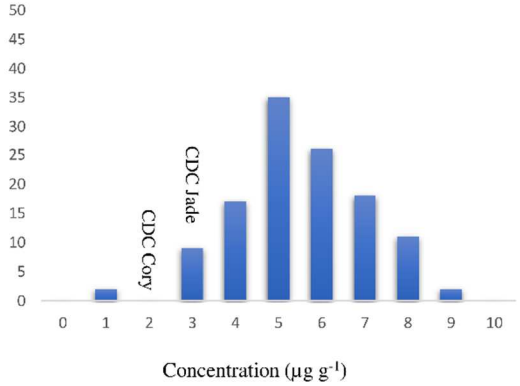
A3: Distribution of violaxanthin in population IJ



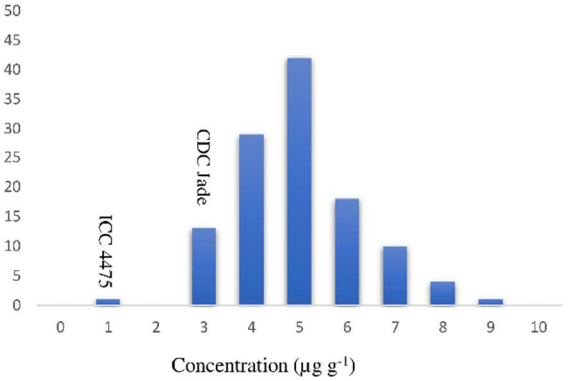
B1: Distribution of zeaxanthin in population JF



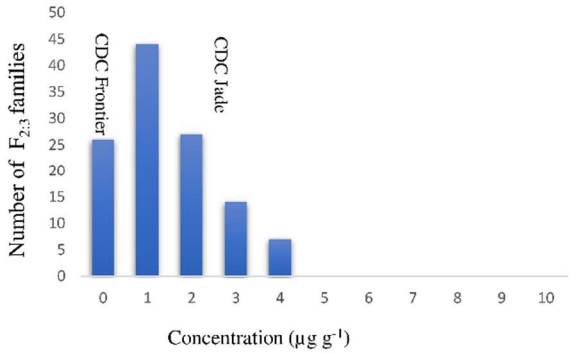
B2: Distribution of zeaxanthin in population CJ



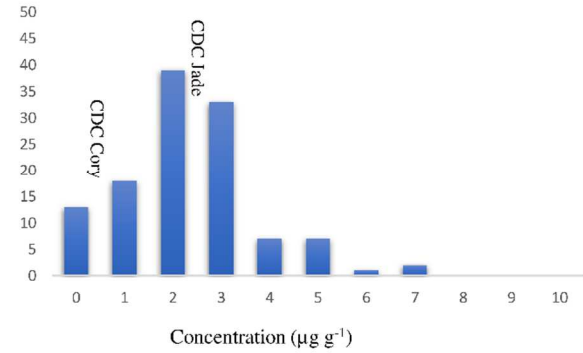
B3: Distribution of zeaxanthin in population IJ



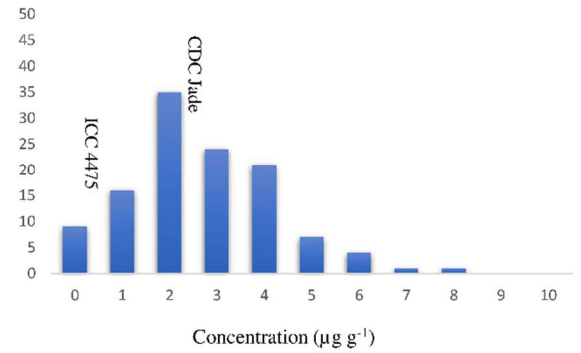
C1: Distribution of β-carotene in population JF



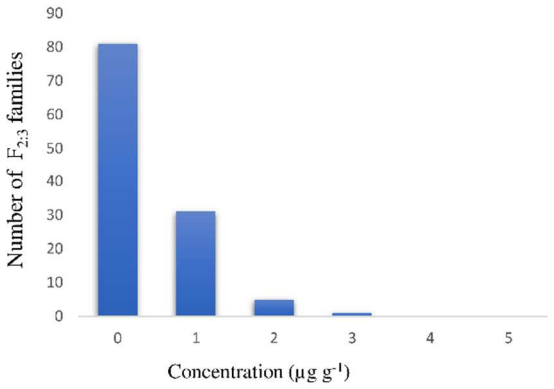
C2: Distribution of β-carotene in population CJ



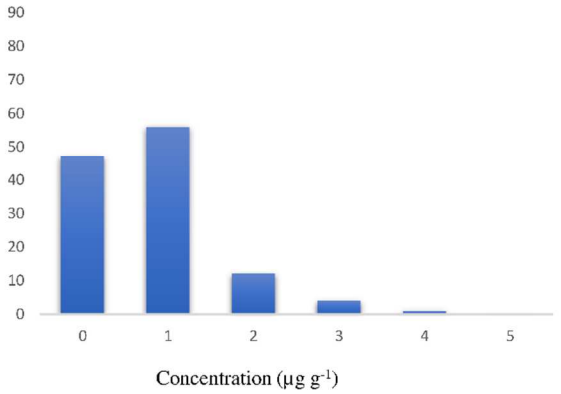
C3: Distribution of β-carotene in population IJ



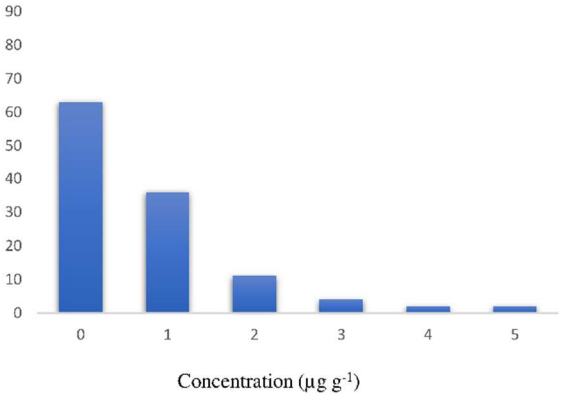
D1: Distribution of β-cryptoxanthin in population JF



D2: Distribution of β-cryptoxanthin in population CJ



D3: Distribution of β-cryptoxanthin in population IJ



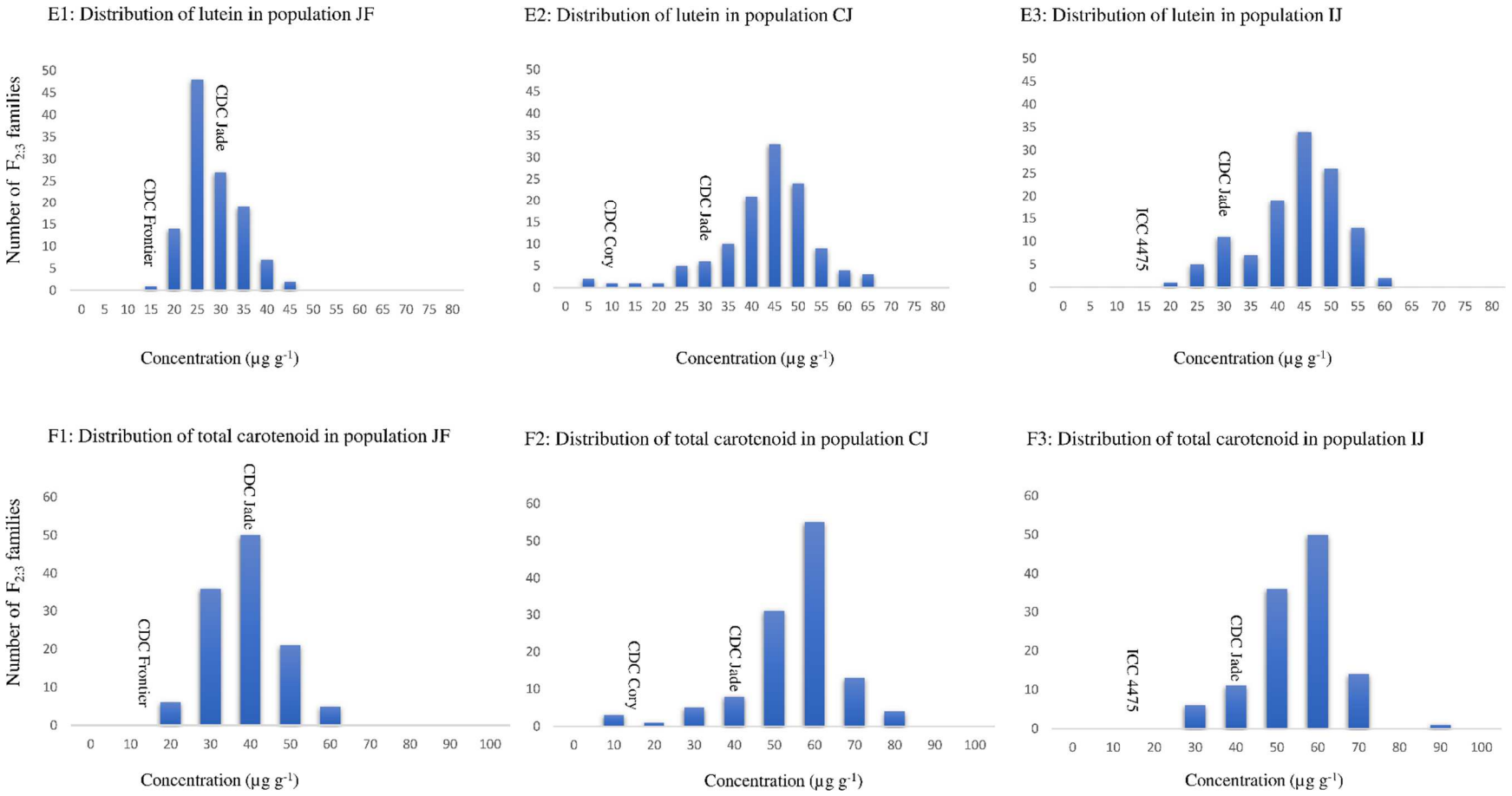


Figure 4.2. Frequency distribution of concentration (µg g^{-1}) of violaxanthin (A), zeaxanthin (B), β-carotene (C), β-cryptoxanthin (D), lutein (E) and total carotenoids (F) measured by HPLC method in three chickpea populations: CDC Jade × CDC Frontier (JF), CDC Cory × CDC Jade (CJ) and ICC4475 × CDC Jade (IJ). The concentration of each carotenoid component for each parent is indicated in the graph. The concentration of β-cryptoxanthin was not detected in the parents.

4.3.3. QTL analysis in three F_2 populations of chickpea

The genotypic data derived from 50 K Axiom® *CicerSNP* chip were examined for missing data and segregation pattern. Those SNP markers which did not fit 1:2:1 ratio, as well as the markers with more than 10% missing data, were removed from further analysis. Based on these filtering, a total of 1,068 bins (4,296 SNPs) were selected for linkage mapping in CDC Jade × CDC Frontier population. The markers were distributed over 8 linkage groups in which the highest (231) and the lowest (62) number of markers were found in linkage group one and linkage group two, respectively. The average of distance between two markers across all linkage groups was 3.07 cM.

Three QTLs were identified on chromosome 1, one each for zeaxanthin (q-Zea-1-JF), β-carotene (q-Crt-1-JF) and lutein concentration (q-Lut-1-JF). An additional QTL was found on chromosome 5 for zeaxanthin (q-Zea-5-JF). Four QTLs were identified on chromosome 8, 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 explained the highest (67%) and the lowest (5%) of phenotypic variation, respectively, in this population. The cotyledon colour (CotCol) was mapped on chromosome 8 overlapping with q-Crt-8-JF and q-Vio-8-JF (Figure 4.3 and Table 4.4).

In CDC Cory × CDC Jade (CJ) population, a total of 694 bins (2,541 SNP markers) were mapped onto 8 linkage groups with one partial linkage group. The maximum number of SNP markers (216) were placed on linkage group four and the minimum number (18) were on linkage group six. The average distance between two markers across all linkage groups was 3.03 cM. Three major QTLs were found on chromosome 8. One QTL was identified for each of β-cryptoxanthin (q-Cryp-8-CJ) and β-carotene (q-Crt-8-CJ). Another genomic region on chromosome 8 was associated with three QTLs (q-Vio-8-CJ, q-Lut-8-CJ and q-Tot-8-CJ) for violaxanthin, lutein and total carotenoids. Each QTL accounted for moderate to a large amount of phenotypic variation in this population. The highest and the lowest PVE was 69% and 23% for β-carotene and total carotenoids, respectively (Figure 4.4 and Table 4.5). QTL results in this population are lacking for the genomic region associated with zeaxanthin concentration.

In population ICC4475 × CDC Jade (CJ), after filtering, 581 bins (2,550 SNP markers) were used for linkage mapping that resulted in 8 linkage groups. The average distance between two markers across all linkage groups was 4.77 cM. One QTL for β-carotene (q-Crt-8-IJ) was

found on chromosome 3 which explained 8% of the phenotypic variation for β-carotene concentration. Four QTLs were found on chromosome 8, 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 colour (Figure 4.5 and Table 4.6). No QTLs were found for zeaxanthin and violaxanthin concentration in this population.

In addition to the CotCol marker, the previously reported stay-green gene (*SGR*) was identified in CDC Jade × CDC Frontier and CDC Cory × CDC Jade populations on linkage group 8 (2047217..2049321) within the QTLs for different carotenoid components. The summary of all identified QTLs and potential candidate genes within the QTL regions associated with carotenoid concentration as well as cotyledon colour are listed in table 4.7 and 4.8, respectively.

Table 4.4. Results of QTL analysis in CDC Jade × CDC Frontier population including the number of QTLs, traits, chromosomal location of each QTL (Chr), map position (Pos), closest marker, bordering markers, LOD values, phenotypic variance explained (PVE) by each QTL, additive (Add) and dominance (Dom) effects and confidence interval (CI, 95%).

QTL	Trait	Chr	Pos (cM)	Closest marker	Left marker	Right marker	LOD	PVE (%)	Add	Dom	CI (cM)
q-Zea-1-JF	Zeaxanthin	1	325	AX-123644659	AX-123618241	AX-123618287	4.1	13	0.60 (CDC Jade)	0.40 (CDC Jade)	323.5 – 327.5
q-Crt-1-JF	β-carotene	1	554	AX-123641029	AX-123617179	AX-123617173	4.1	5	0.28 (CDC Jade)	-0.18 (CDC Frontier)	547.5 – 558.5
q-Lut-1-JF	Lutein	1	618	AX-123638575	AX-123615858	AX-123615414	5.9	21	2.70 (CDC Jade)	-2.90 (CDC Frontier)	611.5 – 625.5
q-Zea-5-JF	Zeaxanthin	5	367	AX-123632228	AX-123640067	AX-123662533	5.9	20	-0.80 (CDC Frontier)	-0.50 (CDC Frontier)	364.5 – 369.5
q-Tot-8-JF	Total	8	168	AX-123657409	AX-123642874	AX-123657408	4.0	13	3.70 (CDC Jade)	0.70 (CDC Jade)	166.5 – 180.5
q-Cryp-8-JF	β-cryptoxanthin	8	176	AX-123637790	AX-123657409	CotCol	5.6	22	0.30 (CDC Jade)	-0.08 (CDC Frontier)	172.5 – 180.5
q-Crt-8-JF	β-carotene	8	177	AX-123657409	CotCol	AX-123637790	34.5	67	1.20 (CDC Jade)	-0.00 (CDC Frontier)	176.5 – 179.5
q-Vio-8-JF	Violaxanthin	8	180	AX-123657409	CotCol	AX-123637790	11.9	37	1.03 (CDC Jade)	-0.25 (CDC Frontier)	176.5 – 183.5

Note: Positive and negative additive and dominance effects indicated increased effects contributed by alleles from CDC Jade and CDC Frontier, respectively.

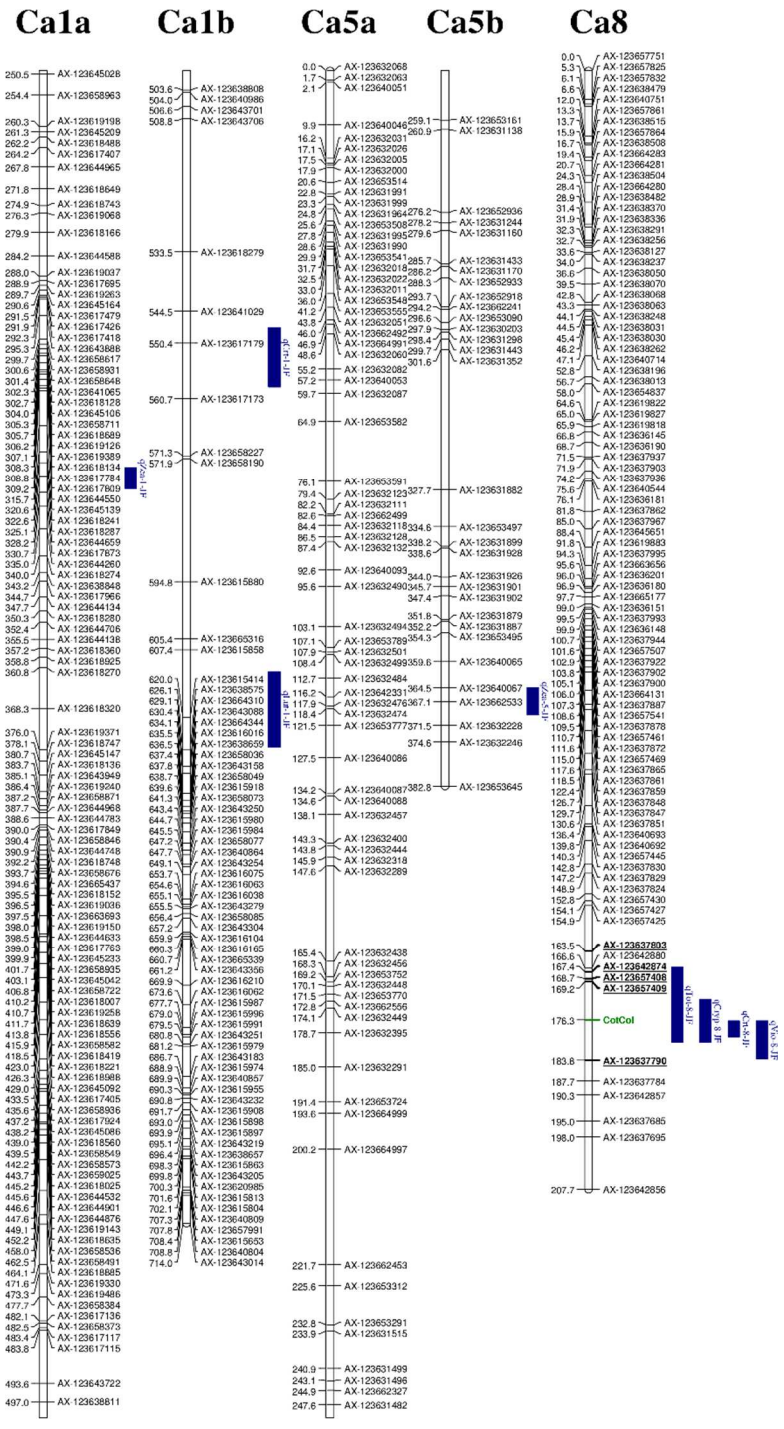


Figure 4.3. Partial linkage map of the F_2 population from a cross between CDC Jade × CDC Frontier showing the QTL associated with carotenoids on linkage groups 1, 5 and 8. The cotyledon colour (CotCol) with major QTL for β-carotene is indicated on Chr 8.

Table 4.5. Results of QTL analysis in CDC Cory × CDC Jade population including the number of QTLs, traits, chromosomal location of each QTL (Chr), map position (Pos), closest marker, bordering markers, LOD values, phenotypic variance explained (PVE) by each QTL, additive (Add) and dominance (Dom) effects, and confidence interval (CI, 95%).

QTL	Trait	Chr	Pos (cM)	Closest marker	Left Marker	Right Marker	LOD	PVE (%)	Add	Dom	CI (cM)
q-Cryp-8-CJ	β-cryptoxanthin	8	97	AX-123642874	AX-123657408	AX-123637792	17.2	45	-0.65 (CDC Jade)	0.03 (CDC Cory)	95.5 – 97.5
q-Crt-8-CJ	β-carotene	8	99	AX-123642869	AX-123637792	CotCol	31.1	69	-1.69 (CDC Jade)	-0.44 (CDC Jade)	97.5 – 100.5
q-Vio-8-CJ	Violaxanthin	8	101	AX-123637792	CotCol	AX-123642869	9.2	33	-0.37 (CDC Jade)	0.63 (CDC Cory)	98.5 – 103.5
q-Lut-8-CJ	Lutein	8	101	AX-123637792	CotCol	AX-123642869	8.3	25	-1.64 (CDC Jade)	9.89 (CDC Cory)	100.5 – 103.5
q-Tot-8-CJ	Total	8	101	AX-123637792	CotCol	AX-123642869	8.3	23	-3.53 (CDC Jade)	10.77 (CDC Cory)	100.5 – 103.5

Note: Positive and negative additive and dominance effects indicated increased effects contributed by alleles from CDC Cory and CDC Jade, respectively.

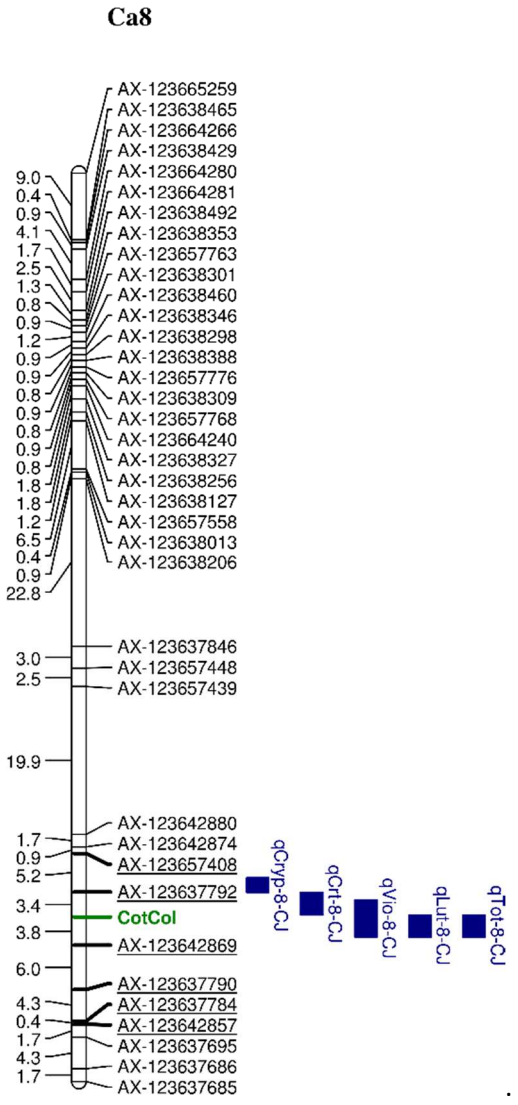


Figure 4.4. Partial genetic map of the F_2 population derived from a cross between CDC Cory × CDC Jade showing the major QTLs and cotyledon colour (CotCol) locus associated with β-cryptoxanthin, violaxanthin, β-carotene, lutein and total carotenoids.

Table 4.6. Results of QTL analysis in ICC4475 × CDC Jade population including the number of QTLs, traits, chromosomal location of each QTL (Chr), map position (Pos), closest marker, bordering markers, LOD values, phenotypic variance explained (PVE) by each QTL, additive (Add) and dominance (Dom) effects, and confidence interval (CI, 95%).

QTL	Trait	Chr	Pos (cM)	Closest marker	Left Marker	Right Marker	LOD	PVE(%)	Add	Dom	CI (cM)
q-Crt-3-IJ	β-carotene	3	201	AX-123646778	AX-123659050	AX-123619520	6.1	8	0.62 (ICC4475)	-0.20 (CDC Jade)	199. –203.5
q-Cryp-8-IJ	β-cryptoxanthin	8	20	AX-123642855	AX-123642846	CotCol	17.4	41	-1.05 (CDC Jade)	-1.05 (CDC Jade)	10.5 – 26.5
q-Crt-8-IJ	β-carotene	8	29	AX-123642855	AX-123642846	CotCol	24.5	58	-1.59 (CDC Jade)	-0.41 (CDC Jade)	25.5 – 29.5
q-Lut-8-IJ	Lutein	8	30	AX-123637737	CotCol	AX-123642855	7.6	24	-0.03 (CDC Jade)	8.16 (ICC4475)	17.5 – 35.5
q-Tot-8-IJ	Total	8	30	AX-123637737	CotCol	AX-123642855	6.3	21	-2.70 (CDC Jade)	7.38 (ICC4475)	20.5 – 34.5

Note: Positive and negative additive and dominance effects indicated increased effects contributed by alleles from ICC4475 and CDC Jade, respectively.

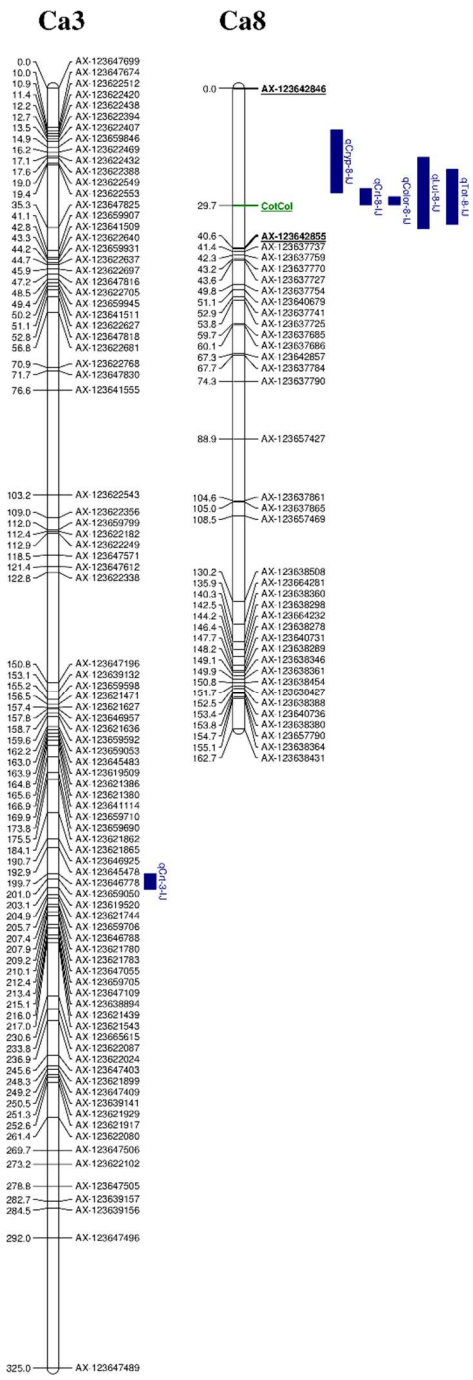


Figure 4.5. Partial genetic map of the F_2 population from a cross between ICC4475 × CDC Jade showing linkage group 3 and 8 that contained QTLs for β-carotene, cotyledon colour, β-cryptoxanthin, lutein and total carotenoids.

Table 4.7. Summary of QTLs associated with violaxanthin, lutein, zeaxanthin, β-carotene, β-cryptoxanthin, total carotenoids and cotyledon colour identified across three F_2 populations: CDC Jade × CDC Frontier, CDC Cory × CDC Jade and ICC4475 × CDC Jade.

Trait	QTLs in CDC Jade × CDC Frontier population	QTLs in CDC Cory × CDC Jade population	QTLs in ICC4475 × CDC Jade population
Violaxanthin	q-Vio-8-JF	q-Vio-8-CJ	
Lutein	q-Lut-1-JF	q-Lut-8-CJ	q-Lut-8-IJ
Zeaxanthin	q-Zea-1-JF and q-Zea-5-JF	-	-
β-carotene	q-Crt-1-JF and q-Crt-8-JF	q-Crt-8-CJ	q-Crt-3-IJ and q-Crt-8-IJ
β-cryptoxanthin	q-Cryp-8-JF	q-Cryp-8-CJ	q-Cryp-8-IJ
Total carotenoids	q-Tot-8-JF	q-Tot-8-CJ	q-Tot-8-IJ

Table 4.8. List of potential candidate genes within the QTL regions associated with different carotenoid components in three F_2 populations: CDC Jade × CDC Frontier (JF), CDC Cory × CDC Jade (CJ) and ICC4475 × CDC Jade (IJ).

Carotenoid component	QTL	Gene ID	Putative function
Lutein	q-Lut-1-JF	LOC101509645	Chlorophyll a-b binding protein CP24 10A chloroplastic
Lutein	q-Lut-1-JF	LOC101493012	Pyruvate decarboxylase 1-like
Lutein	q-Lut-1-JF	LOC101505152	Cytochrome P450 89A2-like
Lutein	q-Lut-1-JF	LOC101502583	Auxin-repressed 12.5 kDa protein
Lutein	q-Lut-1-JF	LOC101513952	Auxin response factor 3-like transcript variant X1, X2 and X3
β-carotene	q-Crt-1-JF	LOC101500378	ABA-responsive protein ABR17-like
β-cryptoxanthin	q-Cryp-8-JF	LOC101509366	Protein STAY-GREEN chloroplastic-like
β-carotene	q-Crt-8-JF	LOC101509366	Protein STAY-GREEN chloroplastic-like
Violaxanthin	q-Vio-8-JF	LOC101509366	Protein STAY-GREEN chloroplastic-like
Total	q-Tot-8-JF	LOC101509366	Protein STAY-GREEN chloroplastic-like
Violaxanthin	q-Vio-8-CJ	LOC101509366	Protein STAY-GREEN chloroplastic-like
β-carotene	q-Crt-8-CJ	LOC101509366	Protein STAY-GREEN chloroplastic-like
Lutein	q-Lut-8-CJ	LOC101509366	Protein STAY-GREEN chloroplastic-like
Total	q-Tot-8-CJ	LOC101509366	Protein STAY-GREEN chloroplastic-like

4.4. Discussion

The current study used three F_2 populations derived from crossing cultivars with different cotyledon and seed coat colours, and different levels of carotenoids (Ashokkumar et al., 2014). CDC Jade was used in each cross as it had higher carotenoid concentration compared to other cultivars in addition to its distinct cotyledon colour (Rezaei et al., 2016). Among the four parental cultivars, CDC Jade had the highest concentration of provitamin A (β-carotene and β-cryptoxanthin) and total carotenoids (3.0 and 45 µg g^{-1}, respectively). Transgressive segregants were observed in the $F_{2:3}$ families for each carotenoid component (Figure 4.2). In case of CDC Jade × CDC Frontier, the highest concentrations of provitamin A and total carotenoids were 7 and 60 µg g^{-1}, respectively, among the progenies. The concentrations of provitamin A and total carotenoids in CDC Frontier were 0.0 and 13.7 µg g^{-1}, respectively. In the second population, (CDC Cory × CDC Jade), the concentrations of provitamin A and total carotenoids were 11 and 80 µg g^{-1}, respectively. In CDC Cory, the concentrations of provitamin A and total carotenoids were 0.1 and 11 µg g^{-1}, respectively. The highest concentrations were observed in ICC4475 × CDC Jade population with 13 and 90 µg g^{-1} for provitamin A and total carotenoids, respectively. In ICC4475, the concentrations of provitamin A and total carotenoids were 0.0 and 13 µg g^{-1} , respectively. Chi-square analysis for cotyledon colour segregation across the five F_2 populations showed the ratio of 3 yellow and 1 green as the best fit for each population. The results confirmed that cotyledon colour is controlled by a single gene, with yellow cotyledon is dominant over the green cotyledon colour. The same result was previously reported in pea (Sato et al., 2007).

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

In 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, while CDC Frontier had the lowest carotenoid concentration. This condition made this population ideal for mapping carotenoids. In addition, the number of bins used for linkage mapping of this population was almost double the other crosses. The identification of the largest number of QTLs in this population is due to high genetic variation in the progeny.

Cotyledon colour (CotCol) as a monogenic trait was mapped on to chromosome 8 in each population. The usefulness of the cotyledon colour as a morphological marker was demonstrated in faba bean (Cruz-Izquierdo et al., 2012). The majority of the QTLs for different carotenoids were located in the vicinity of the CotCol locus on the map. Clustering of QTLs for carotenoids is likely due to the clustering of the carotenoid genes in the genome (Santos and Simon, 2002). However, the exact positions of the common QTLs varied slightly among the three populations likely due to different map density among the populations (Long et al., 2008). The *SGR* gene was found within the QTLs for violaxanthin (q-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 CDC Jade x CDC Frontier population. The *SGRs* are responsible for chlorophyll break down. Mutation in this gene leads to stay green phenotype (Ren et al., 2007a; Sato et al., 2007). Pea plants containing mutant allele (*sgr*) produced green mature seeds and their leaves retained the greenness during senescence (Barry et al., 2008). We observed the same situation in our green chickpea cultivars CDC Jade and CDC Verano. It was confirmed by Rezaei et al. (2016) that the carotenoid concentration decreased as the seeds matured, but in green cotyledon cultivars, the reduction rate was slower than in the yellow cotyledon chickpeas. Previous report showed that a combination of chlorophyll and carotenoids increased antioxidant activities (Sgherri et al., 2015) and resulted in higher yield (Thomas and Howarth, 2000). The high stability of light harvesting complex (LHC; Sato et al., 2007) and low cleavage and hydroxylation activities (Rezaei et al., 2016) were responsible for the elevated carotenoid concentration in the green cotyledon chickpeas. In CDC Cory × CDC Jade population, four QTLs for violaxanthin (q-Vio-8-CJ), lutein (q-Lut-8-CJ), β-carotene (q-Crt-8-CJ), and total carotenoids (q-Tot-8-CJ) co-localized with the *SGR*. It is important to note that lutein and β-carotene are common carotenoids in photosystem of green tissues. The absence of chlorophylls break down, which resulted in green colour, elevated provitamin A concentration in these populations. The highest PVE in all three populations was obtained from QTLs for β-carotene concentration as follow: q-Crt-8-CJ (69% PVE), q-Crt-8-JF (67% PVE), and q-Crt-8-IJ (58%

PVE). These QTLs are very important, as they are present in all three populations for β-carotene and all closely linked to CotCol. It showed that a common genomic region controls both cotyledon colour and β-carotene concentration and this region might be critical for the marker-assisted selection (MAS) in chickpea breeding for provitamin A improvement.

The carotenoid profile in the $F_{2:3}$ seeds is in agreement with the results of the analysis using the diverse chickpea panel presented in chapter 3, however, the concentration of all five carotenoid components were higher in the biparental mapping populations. The highest improvement was observed in β-carotene and β-cryptoxanthin levels. It seemed that the allele from CDC Jade which was used as either female or male parent in all three populations is responsible for elevated provitamin A concentration. The results that the cotyledon colour is highly associated with provitamin A concentration suggested that the measurement of colour density in chickpeas may work for quick detection of provitamin A in the seeds. In case of wheat, two factors including flour yellow colour (b^*) and yellow pigment content (YPC) were used as an alternative to the costly HPLC method to detect carotenoids (Zhai et al., 2016). The visual selection for carotenoids has been successfully practiced in maize (Burt et al., 2013) and it is reported that the dark orange endosperm is a target trait in breeding for provitamin A biofortification in maize (Owens et al., 2014). In the GWAS, the gene pyruvate decarboxylase 1 was significantly associated with β-carotene in the chickpea diverse panel. The same gene was found within the QTL q-Lut-1-JF for the lutein concentration in CDC Jade x CDC Frontier population. One member of P450 family was co-located with QTL q-Lut-1-JF for lutein (Table 4.8). The P450 gene families were also associated with provitamin A concentration in our GWAS. Overall, at least one QTL with PVE of more than 13% was found for each carotenoid component in each population. Based on the results obtained in this study, QTLs with moderate to large effects control the carotenogenesis in chickpea. Similar findings were 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 including CDC Jade × CDC Frontier, CDC Cory × CDC Jade, and ICC4475 × CDC Jade. The QTLs responsible for the concentration of various carotenoids such as violaxanthin, lutein, zeaxanthin, β-carotene, and β-cryptoxanthin were identified in this study. The cotyledon colour as a monogenic trait was mapped on chromosome 8 in all three populations. In addition, the stay green gene (*SGR*) was co-localized with QTLs for violaxanthin (q-Vio-8-JF), β-carotene (q-Crt-8-JF), β-cryptoxanthin (q-Cryp-8-JF), and total carotenoids (q-Tot-8-JF) in CDC Jade by CDC

Frontier population as well as QTLs for violaxanthin (q-Vio-8-CJ), lutein (q-Lut-8-CJ), β-carotene (q-Crt-8-CJ), and total carotenoids (q-Tot-8-CJ) in CDC Cory x CDC Jade population. The majority of the QTLs associated with carotenoids were located on chromosome 8 close to the genomic region associated with the cotyledon colour in all three F_2 populations. A significant positive relationship between cotyledon colour and carotenoid concentration was found in this study. Crossing of chickpea cultivars with green and yellow cotyledons was found to be an effective way to map carotenoid components, especially for the provitamin A concentration. The QTLs that accounted for high PVE, have the potential for use as markers in breeding programs.

CHAPTER 5. CAROTENOID BIOSYNTHESIS IN CHICKPEA SEEDS: CANDIDATE GENES IDENTIFICATION, GENOME STRUCTURE ANALYSIS AND EXPRESSION PATTERN

Preface

The last objective of my doctoral research project is addressed in the following chapter with the focus on the analyses of structure, domains and copy numbers of genes responsible for carotenoid biosynthesis as well as their expression patterns during seed development in chickpea. The experiment was conducted in 2014 under greenhouse conditions using five chickpea cultivars with different seed coat and cotyledon colours. Genes from the carotenoid pathway were retrieved from the chickpea genome assembly and SNP variations for the selected genes were anlyzed among five chickpea cultivars. The carotenoid measurement was conducted as described in chapter 3 and 4. Gene expression analysis was done using quantitative PCR at four stages post anthesis in the chickpea seeds. Results showed that the expression of the genes involved in the carotenoid pathway and carotenoid concentration were higher at early phases after anthesis then decreased during seed maturation. Also, chickpea carotenoids were affected by mutations and the structure of the carotenoid genes in this study.

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Author contributions: Mohammad Kazem Rezaei conducted the experiments, analyzed and summarized the results. Mohammad Kazem Rezaei, Amit Deokar, and Bunyamin Tar'an wrote and finalized the manuscript; Bunyamin Tar'an conceived and directed the project.

5.1. Introduction

Chickpea (*Cicer arietinum* L.) is one of the most important legume crops in the semi-arid tropics. Its worldwide production ranks second after common bean (FAOSTAT, 2012). It is considered as one of the most important food legumes in the developing countries because of its nutritional value and its capacity for symbiotic nitrogen fixation that can provide the entire crop demand for nitrogen (Jukanti et al., 2012). Two chickpea types kabuli and desi, are commonly grown. Selection for traits like flower colour and zero tannins in seed resulted in the evolving kabuli from desi chickpea in the Mediterranean basin (Moreno and Cubero, 1978; Jana and Singh, 1993; Khan et al., 2010). Chickpea has a relatively small (740 Mb) diploid (2n= 2x= 16) genome (Arumuganathan and Earle, 1991) and the genome sequence of both kabuli (Varshney et al., 2013a) and desi (Jain et al., 2013) types are available that makes it a good case for legume genetic and genomic research. Chickpea is a good source of vitamins including riboflavin, niacin, thiamin and β-carotene as the precursor of vitamin A (Cabrera et al., 2003; Abbo et al., 2005). Vitamin A deficiency leads to xerophthalmia that causes blindness among children (World Health Organization, 2009). The deficiency also increases the chance of getting malaria and diarrheal disease (ACC/SCN, 2000). Carotenoids are categorized as a group of lipophilic yellow, orange, and red pigments primarily produced by photosynthetic organisms and also by certain fungi and bacteria (Khoo et al., 2011). They play a key role in photosynthesis and prevent photooxidation damage in plants (Howitt and Pogson, 2006). There are two major classes of carotenoids: i) oxygenated (or xanthophyll) that includes lutein, violaxanthin, and neoxanthin and (ii) non-oxygenated (or carotenes) that include β-carotene and lycopene (Dellapenna and Pogson, 2006). Carotenoid is one of the products of isoprenoid pathway and its biosynthetic pathway is conserved in plants (Dellapenna and Pogson, 2006).

Plant carotenoids are C40 isoprenoid compounds that are produced through two separate pathways: plastid-localized 2-C-methyl-D-erythritol 4-phosphate (MEP) and cytoplasmic mevalonate pathway. The MEP pathway is responsible for producing a large number of carbon flux that used for carotenoids biosynthesis (Giuliano, 2014). In the first step of MEP pathway, 1-deoxy-D-xylulose 5-phosphate (DXP) is derived from pyruvate and glyceraldehydes-3-phosphate under the control of 1-deoxy-D-xylulose-5-phostaphate synthase (*DXS*). The conversion of DXP to MEP is performed by 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*DXR*; Julliard and Douce, 1991; Julliard, 1992). In the following steps, production of isopentenyl diphosphate (IPP)

and dimethylallyl diphosphate (DMAPP) is mediated by 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase (*HDR*; Lichtenthaler, 1999). Then, three molecules of IPP and one molecule of DMAPP are condensed into geranyl-geranyl diphosphate (GGPP) by GGPP synthase (*GGPPS*; Kleinig, 1989). Two molecules of GGPPs are converted to phytoene by phytoene synthase (*PSY*; Rodríguez-Concepción, 2010). Subsequently, phytoene is converted into lycopene through four desaturation and two isomerization reactions (Bartley et al., 1991; Albrecht et al., 1995; Chen et al., 2010; Yu et al., 2011). Lycopene cyclization is facilitated by two enzymes lycopene β-cyclase (*LCYB*) and lycopene ε-cyclase (*LCYE*) that finally produce β-carotene and α-carotene from lycopene (Pogson et al., 1996; Owens et al., 2014). The two molecules β-carotene and α-carotene are then converted to zeaxanthin (β, β-carotene 3,3´-diol) and lutein (β, α-carotene 3,3´-diol), respectively by β-carotene hydroxylase (Goodwin, 1993). The conversion of zeaxanthin into violaxanthin is mediated by enzyme zeaxanthin epoxidase (*ZEP*; Misra et al., 2006; Chen et al., 2014b). Carotenoids are also precursors for apocarotenoids such as plant hormone abscisic acid, which is essential for plant growth and development (Kermode, 2005; Umehara et al., 2008).

Apocarotenoid formation is mediated by carotenoid cleavage dioxygenases 1 (*CCD1*; Auldridge et al., 2006). Violaxanthin de-epoxidase works as part of the xanthophyll (or violaxanthin) cycle and has a key role on the de-epoxidation of xanthophyll pigments such as violaxanthin (V) and antheraxanthin (A) into zeaxanthin (Z; Misra et al., 2006; Chen et al., 2014b). In the last step, violaxanthin is converted to allenic carotenoid neoxanthin by neoxanthin synthase (*NXS*; Welsch et al., 2008). Each class of these enzymes seems to be responsible for the carotenoid metabolism at different and specific subcellular sites under both normal and stress conditions (Rubio et al., 2008). Specific gene family members that are responsible for carotenoid content and composition during endosperm development have been well characterized in maize (Li et al., 2008; Vallabhaneni et al., 2009; Vallabhaneni and Wurtzel, 2009). Various approaches including metabolic and genetic engineering (Welsch et al., 2010; Kumar et al., 2012; Mintz-Oron et al., 2012; Nogueira et al., 2013; Ariizumi et al., 2014), advanced genomics and bioinformatics (Wurtzel et al., 2012), genomic-assisted selection (Campbell et al., 2014; Owens et al., 2014) and transcriptome analysis (Caroca et al., 2013; Frusciante et al., 2014) have been applied for studying carotenogenesis and improvement of carotenoid content in different crops. To develop chickpea cultivars with increased carotenoid concentration, information on the genetic basis of carotenogenesis in chickpea is substantial. The availability of genome assembly of chickpea (Jain

et al., 2013; Varshney et al., 2013a) provides a good source of information to retrieve the potential candidate genes involved in carotenogenesis in chickpea.

The objectives of this research were first to identify candidate genes for carotenogenesis in chickpea through genome-wide analysis, and secondly to examine their expression pattern and their correlation with carotenoid concentration at different developmental stages of chickpea seeds from the representative genotypes.

5.2. Materials and methodology

5.2.1. Candidate gene analysis

The sequences of genes involved in the carotenoid pathway were collected from *Medicago truncatula* and *Arabidopsis thaliana* genomes in the GeneBank database such as National Center for Biotechnology Information (NCBI BLAST® online database). The sequences were blasted against CDC Frontier genome assembly (Varshney et al., 2013a) in order to retrieve the gene sequences from chickpea. The structure of the genes and proteins in the carotenoid and isoprenoid pathways were analyzed in chickpea, *Medicago truncatula* and *Arabidopsis thaliana*. To evaluate the similarity of the genes that have more than two copy numbers, protein sequence alignment was done using Bio Edit sequence alignment editor software (Hall, 1999).

5.2.2. Re-sequencing and SNP calling

Whole genome resequencing was done on four chickpea cultivars CDC Verano, CDC 441-34, CDC Jade and CDC Cory. The Paired-end (PE) genomic DNA libraries were constructed from 1 µg of gDNA using Illumina TruSeq DNA PCR-Free HT Library Preparation Kits (Illumina, Inc) and sequenced on Illumina HiSeq 2500 using 2 x 125 chemistry.The raw data were subjected to filtration and correction steps using Trimmomatic v0.35 (Bolger et al., 2014). The obtained high-quality reads were then aligned to CDC Frontier reference genome sequence using BWAv0.7.12 (Li, 2013), and finally, variant calling was performed using GATK v3.5 HaplotypeCaller pipeline (McKenna et al., 2010). We selected 32 genes from both carotenoid and isoprenoid pathways for SNP analysis. The genome assembly of CDC Frontier was considered as the reference and the sequences from the other four cultivars were compared to the CDC Frontier genome annotation. The 2kb upstream and downstream of each gene sequence were chosen to cover the intergenic region for SNP discovery. Using SnpEff software, the SNPs among the five chickpea cultivars were identified and extracted (Cingolani et al., 2012).

5.2.3. Seed sample collection

Five chickpea cultivars with different cotyledon colours including CDC Frontier (yellow cotyledon kabuli), CDC Verano (green cotyledon kabuli), CDC 441-34 (yellow cotyledon kabuli), CDC Jade (green cotyledon desi), and CDC Cory (yellow cotyledon desi) were grown in 15 L pots filled with Sunshine mix #4 media (SunGrow, Seba Beach, Alberta, Canada) in the greenhouse at the University of Saskatchewan in 2014. The NPK fertilizer (20-20-20) was added to each pot (three g L^{-1}) for three times after plants reached the height of 20 cm. All plants were grown under controlled condition in the greenhouse with average 21.8 °C air temperature, 72% humidity and 17 hours of lights and 7 hours of darkness.

The flowers were tagged at the day of anthesis (before the flowers open completely) and 3 to 4 developing seeds (pods) were harvested at each growth stage at 8, 16, 24 and 32 days post-anthesis (DPA). Three biological replicates were planted for each cultivar and seed growth stage. Harvested pods were immediately floated in liquid nitrogen and were kept in -80 °C until RNA extraction.

5.2.4. Gene expression analysis

Different primer pairs were designed using Primer3 online program (www.primer tool; Whitehead Institute for Biomedical Research, 1998) for q-PCR analysis and their accuracy was checked by the Primer-BLAST program (Table A10). Total RNA was isolated from chickpea seeds of four developmental stages using hexadecyltrimethylammonium bromide (CTAB) according to the procedure described by Kannan et al. (2014). Extracted RNA samples were treated with DNase I (Life Technology, Invitrogen, USA) to remove any DNA contamination. The synthesis of first cDNA strand was performed using Sensi FAST™ cDNA Synthesis Kit (BIOLINE, USA). The real-time PCR assay was conducted using C1000 Touch™ Thermal Cycler (BIO-RAD, USA) using the Sensi FAST™ SYBR NO-ROX Kit (BIOLINE, USA). To estimate primer efficiency the PCR product amplified by each primer was purified using QIAquick® PCR Purification Kit and a serial dilution for each individual product directly used in qPCR assay. In the second method, a serial dilution was made based on cDNA and then an equation was calculated based on different cDNA dilution and cycle threshold (ct) corresponded to each concentration. The calculated slope from equation for each primer was used in the formula below for determination of primer efficiency. $e = (10^{1/slope} - 1) \times 100$

The relative expression was calculated using $2^{(-\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001). In order to find the most appropriate internal control, six housekeeping genes including actin 1 (*Act1*), elongation factor 1-alpha (*Ef1α*), glyceraldehyde-3-phosphate dehydrogenase(*GAPDH*), initiation factor 4a (*IF4a*), heat shock protein 90 (*HSP90*), and 18S ribosomal RNA (*18SrRNA*; Table A10) were selected and examined for their expression (Garg et al., 2010). The 8 DPA stage of CDC Frontier was used as the reference sample and two technical replicates were used for each biological replication to minimize sampling errors. We applied UPMG method to develop a dendrogram based on K-means clustering with Cluster v3.0 program (Eisen et al., 1998). The gene expression patterns are presented as a heat map using Treeview v1.60 (Page, 1996). The gene expression levels are also presented as bar graphs (Figure A2).

5.2.5. Carotenoid measurement

The seed carotenoid was measured in three developmental stages including 16, 24 and 32 DPA in five chickpea cultivars using high-performance liquid chromatography (HPLC). We used 100 mg of fine powder from whole chickpea seed for carotenoid extraction. Extraction and carotenoid analysis performed as described by Rezaei et al., (2016).

5.2.6. Statistical analysis

Pearson correlation analysis was done between transcript levels and carotenoid concentrations in chickpea seeds at different developmental stages. All the statistical analyses were done using SAS software (Version 9.1, SAS Institute Inc., Cary, NC, USA).

5.3. Results

5.3.1. Carotenoid biosynthesis genes in chickpea, Arabidopsis and Medicago

The total number of genes for both isoprenoid and carotenoid pathways were 32 in chickpea and 26 in Arabidopsis. We found more copy numbers of some genes from both pathways in chickpea compared to Arabidopsis which can be explained by the differences in the genome size of these two species. Chickpea genome size of 740 Mbp (Varshney et al., 2013a) is over five times larger than Arabidopsis (135 Mbp; "Arabidopsis Genome Initiative" 2000).

The properties of carotenoid and isoprenoid genes in *Medicago truncatula* were similar to chickpea as both species share common legume family. Except for three genes, *PSY*, *BCH* and *ZEP*, we found similar copy numbers for all carotenoid and isoprenoid genes in *Medicago truncatula* and

in chickpea (Table A7). The estimated genome size of *Medicago truncatula* is 465 Mbp that is bigger than that of *Arabidopsis* (Bennett and Leitch, 2011).

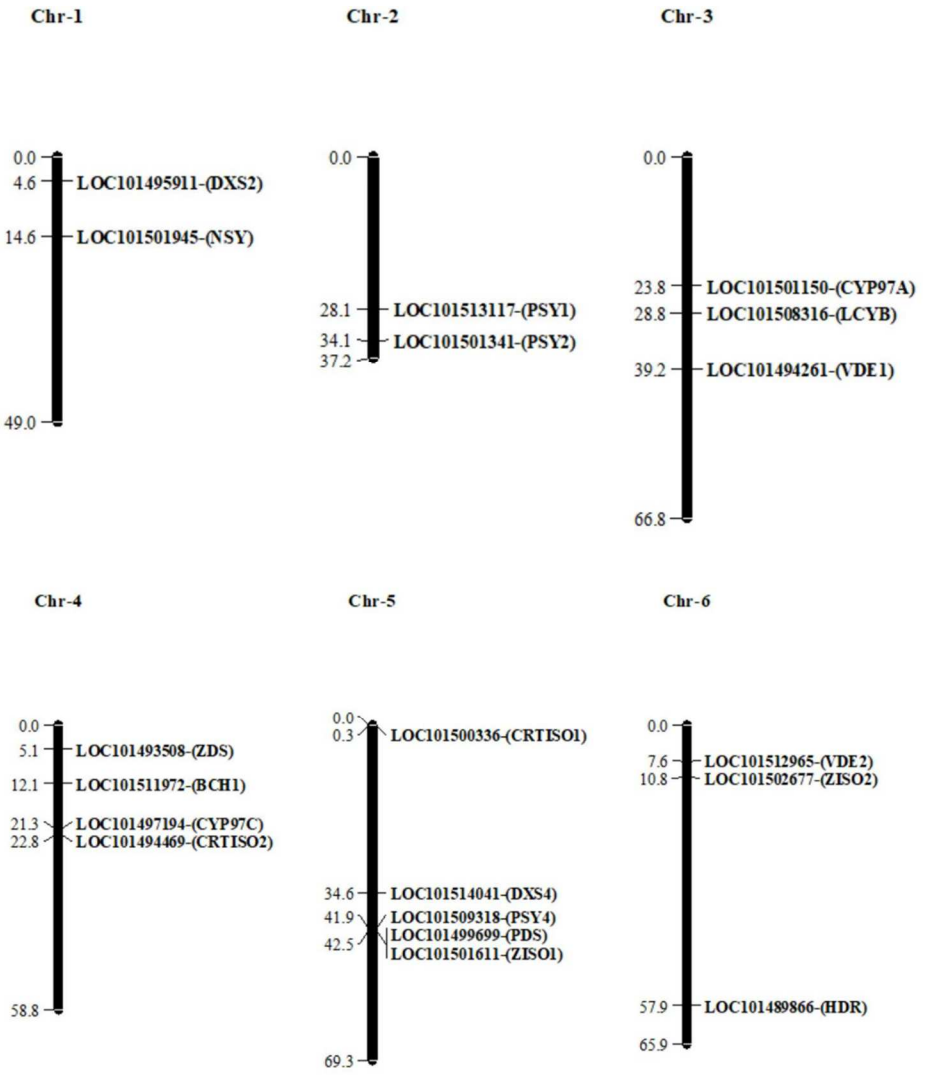
In chickpea, the genes *CCD1* and *GGPPS2* represent the largest and smallest size with 15 Kbp and 1.8 Kbp respectively. Four genes including *GGPPS1*, *GGPPS2*, *LCYB* and *NSY* had only one exon, whereas *ZEP1* and *ZEP2* had the highest number of exons (16 exons). In Arabidopsis, the largest and smallest genes were *PDS* with 6.6 Kbp and *GGPPS2* with 1.7 Kbp, respectively. Two genes *GGPPS1* and *GGPPS2* had only one exon, while *ZEP1* was detected to have the highest number of exons (16 exons) in Arabidopsis (Table A7). Domain analysis showed that the largest number of genes in the pathways have the Rossmann-fold NAD(P)H/NAD(P)(+) binding (NADB) domain in their structure. Interestingly the main domain in *PSY* genes is Isoprenoid Biosynthesis Enzymes- Class 1 that is like *GGPPS* genes in isoprenoid pathway which shows how these two pathways are connected. The properties of all the domains are listed in Table A8. Two genes including *DXS* and *PSY* each with four copy numbers were chosen for sequence similarity analysis in chickpea. Sequence alignment and similarity matrix indicated that *DXS1* and *DXS2* had the highest similarity (0.842) and *DXS4* had the lowest level of similarity with other *DXS* genes. In the case of *PSY* genes, *PSY2* and *PSY3* were highly similar (0.816) and *PSY1* and *PSY4* had the lowest similarity (0.484) within this group (Figure A1 and Table A9).

5.3.2. Sequence analysis and SNP identification

Sequence analysis was done on the 32 candidate genes (Figure 5.1) that play a key role in carotenoid biosynthesis. A total of 476 SNPs were found in the upstream, exon, intron and downstream sequences of the genes across the five chickpea cultivars. A total of 17 SNPs (Table 5.1) was found in the coding region of 1-deoxy-D-xylulose-5-phosphate synthase (*DXS1* and *DXS2*), lycopene β-cyclase (*LCYB*), β-carotene hydroxylase (*BCH1*), zeta carotene isomerase (*ZISO2*), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*DXR2*) and geranyl-geranyl diphosphate synthase (*GGPPS2*). Only two missense mutations, which code for different amino acid, were found in *ZISO2*, while the rest of the SNPs were synonymous mutation. Consequently, the amino acid Serine (S) changed to Proline (P) at position 15 and Phenylalanine (F) to Leucine (L) at position 147 in CDC Verano compared to the reference genome CDC Frontier (Figure 5.2).

Table 5.1. The list of 17 SNPs found in the coding region of 1-deoxy-D-xylulose-5-phostaphate synthase (*DXS1* and *DXS2*), lycopene β-cyclase (*LCYB*), β-carotene hydroxylase (*BCH1*), zeta carotene isomerase (*ZISO2*), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*DXR2*) and geranyl-geranyl diphosphate synthase (*GGPPS2*) with their chromosomal location, physical position (bp), and their differences in reference (CDC Frontier) and alternative annotation (CDC 441-34, CDC Jade, CDC Cory and CDC Verano).

Gene	Chromosome	Position (bp)	Reference	Alternative	CDC 441-34	CDC Jade	CDC Cory	CDC Verano
DXS2	Ca1	4631533	G	A	1/1	1/1	1/1	1/1
LCYB	Ca3	28752373	A	G	1/1	1/1	1/1	1/1
BCH1	Ca4	12093051	T	C	0/0	1/1	0/1	0/1
ZISO2	Ca6	10805615	C	T	0/0	0/0	0/0	0/1
ZISO2	Ca6	10805725	T	G	0/0	0/0	0/0	0/1
ZISO2	Ca6	10805905	C	A	0/0	0/0	0/0	0/1
ZISO2	Ca6	10805923	C	T	0/0	0/0	0/0	0/1
DXS1	Ca7	5529110	A	G	0/0	1/1	1/1	0/1
DXS1	Ca7	5529191	A	G	0/0	1/1	1/1	0/0
DXR2	Ca7	30117254	T	C	1/1	1/1	0/0	1/1
DXR2	Ca7	30117353	A	G	1/1	1/1	0/1	1/1
DXR2	Ca7	30117362	A	G	1/1	1/1	0/1	1/1
DXR2	Ca7	30117491	C	T	1/1	1/1	0/1	1/1
DXR2	Ca7	30117821	T	C	1/1	1/1	0/1	1/1
DXR2	Ca7	30136051	A	G	1/1	1/1	0/0	1/1
DXR2	Ca7	30136328	G	A	1/1	1/1	0/1	1/1
GGPPS2	Ca8	4790033	C	T	0/0	0/1	0/0	0/1


Chr-1
0.0
4.6
LOC101495911-(DXS2)
14.6
LOC101501945-(NSY)
49.0
Chr-2
0.0
28.1
LOC101513117-(PSY1)
34.1
LOC101501341-(PSY2)
37.2
Chr-3
0.0
23.8
LOC101501150-(CYP97A)
28.8
LOC101508316-(LCYB)
39.2
LOC101494261-(VDE1)
66.8
Chr-4
0.0
5.1
LOC101493508-(ZDS)
12.1
LOC101511972-(BCH1)
21.3
LOC101497194-(CYP97C)
22.8
LOC101494469-(CRTISO2)
58.8
Chr-5
0.0
0.3
LOC101500336-(CRTISO1)
34.6
LOC101514041-(DXS4)
41.9
LOC101509318-(PSY4)
LOC101499699-(PDS)
42.5
LOC101501611-(ZISO1)
69.3
Chr-6
0.0
7.6
LOC101512965-(VDE2)
10.8
LOC101502677-(ZISO2)
57.9
LOC101489866-(HDR)
65.9

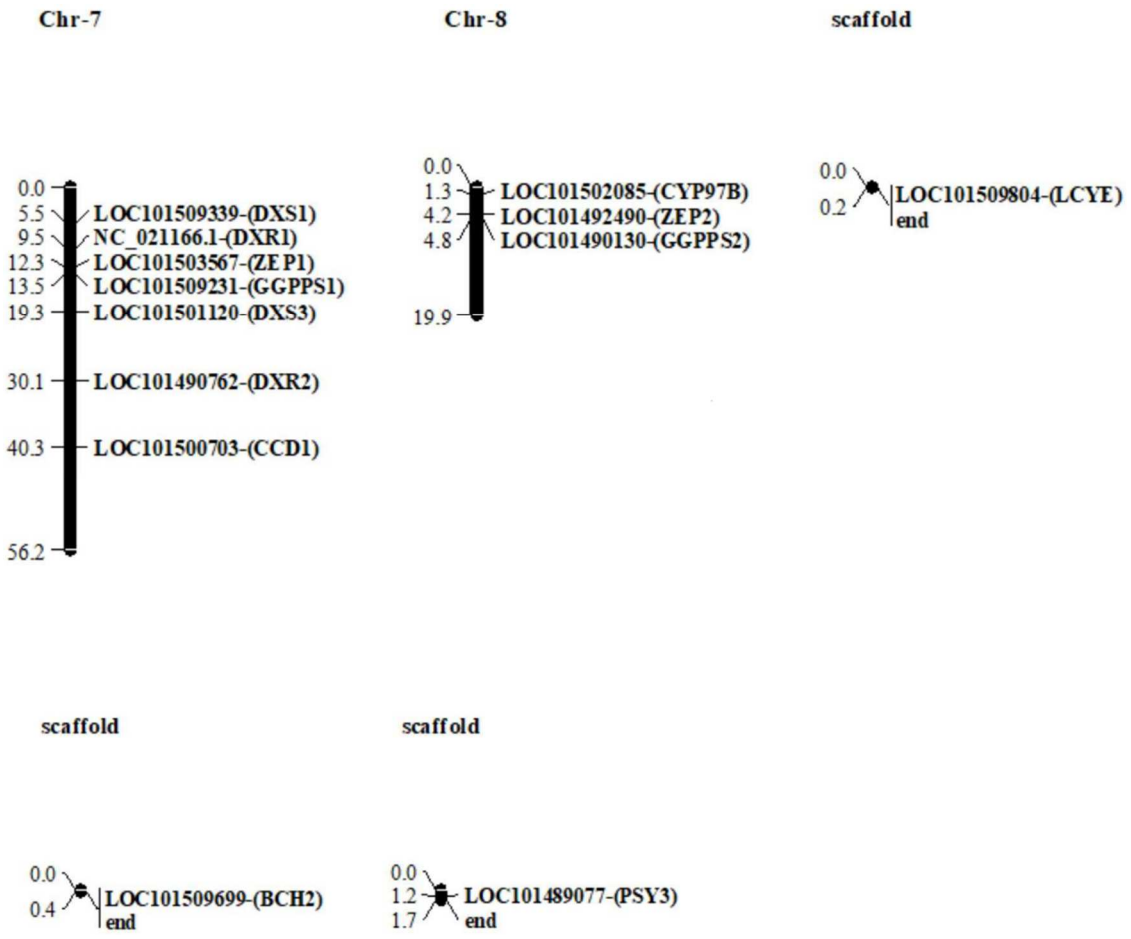


Figure 5.1. Chickpea carotenogenesis genes including 1-deoxy-D-xylulose-5-phostaphate synthase (*DXS1, DXS2, DXS3* and *DXS4*), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*DXR1* and *DXR2*), 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (*HDR*), geranyl-geranyl diphosphate synthase (*GGPPS1* and *GGPPS2*), Phytoene synthase (*PSY1, PSY2, PSY3* and *PSY4*), Phytoene desaturase synthase (*PDS*), 15-cis-zeta-carotene isomerase (*ZISO1* and *ZISO2*), ζ-carotene desaturase (*ZDS*), prolycopene isomerase (*CRTISO1* and *CRTISO2*), lycopene β-cyclase (*LCYB*), lycopene ε-cyclase (*LCYε*), β-carotene hydroxylase (*BCH1* and *BCH2*), cytochrome P450-type monooxygenase (*CYP97A*, *CYP97B*, *CYP97C*), zeaxanthin epoxidase (*ZEP1* and *ZEP2*), violaxanthin de-epoxidase (*VDE1* and *VDE2*), crotenoid 9,10(9',10')-cleavage dioxygenase 1 (*CCD1*), and neoxanthin synthase (*NSY*) with their name, accession number, chromosome location (Ca), and size (kb) that were analyzed in this study. Genes in this figure were retrieved from chickpea assembly genome in both kabuli (Varshney et al., 2013) and desi (Jain et al., 2013) cultivars.

Figure 5.2. Missense mutation in gene *ZISO2* is resulted in change of amino acid Serine (S) to Proline (P) in position 15 and Phenylalanine (F) to Leucine (L) in position 147. This change happened in cultivar CDC Verano (alternative) and the rest of cultivars had similar sequences like reference assembly CDC Frontier.

5.3.3. Carotenoid concentration

The highest concentration of lutein was observed in CDC Jade at 16 DPA. The lutein concentration dropped significantly at 24 and 32 DPA. The lowest concentration of lutein was observed in CDC Frontier. The concentration of zeaxanthin was highest in CDC Cory followed by CDC Jade. The zeaxanthin concentration was lowest and similar in three cultivars CDC Frontier, CDC 441-34 and CDC Verano (Table 5.2). The highest concentration of β-carotene was observed in CDC Cory and CDC Jade at 16 DPA, but at the later stage, we observed the highest concentration in CDC Jade and CDC Verano. CDC Jade and CDC 441-34 showed the highest and lowest concentration of β-cryptoxanthin, respectively, in almost all stages. In addition, the highest and lowest concentration of violaxanthin was observed in CDC Jade and CDC Frontier, respectively. The highest and lowest total carotenoids were observed in CDC Jade and CDC Frontier respectively (Table 5.2). In general, the concentration of several types of carotenoid decreased from 16 to 32 DPA in all cultivars. Overall in the five cultivars, the concentration of lutein was the highest followed by zeaxanthin, β-carotene, β-cryptoxanthin and violaxanthin (Table 5.2).

Table 5.2. Concentration (µg g^{-1}) of different seed carotenoid including lutein, zeaxanthin, β-carotene, β-cryptoxanthin, violaxanthin and total carotenoids ± Se in five chickpea cultivars (CDC Frontier, CDC 441-34, CDC Verano, CDC Cory, and CDC Jade) at 16, 24 and 32 days post-anthesis (DPA).

Accessions	DPA	Lutein	Zeaxanthin	β-Carotene	β-Cryptoxanthin	Violaxanthin	Total
CDC Frontier	16	26.5 ± 1.01	18.25 ± 0.19	2.15 ± 0.16	2.15 ± 0.01	1.32 ± 0.01	50.37
CDC Frontier	24	15.4 ± 0.08	10.1 ± 0.07	1.73 ± 0.19	0.94 ± 0.03	0.66 ± 0.03	28.85
CDC Frontier	32	11.71 ± 0.14	8.74 ± 0.19	1.68 ± 0.14	0.79 ± 0.15	0.68 ± 0.15	23.64
CDC 44134	16	27.43 ± 1.82	20.33 ± 0.68	1.92 ± 0.3	1.18 ± 0.18	1.8 ± 0.18	52.69
CDC 44134	24	17.86 ± 0.35	14.44 ± 0.19	1.86 ± 0.15	0.98 ± 0.0	1.48 ± 0.01	36.63
CDC 44134	32	10.64 ± 0.81	8.1 ± 0.33	1.74 ± 0.08	0.94 ± 0.01	0.68 ± 0.01	22.12
CDC Verano	16	37.43 ± 1.09	29.51 ± 0.16	2.25 ± 0.08	2.3 ± 0.15	2.1 ± 0.15	73.59
CDC Verano	24	19.01 ± 0.45	19.82 ± 0.06	1.78 ± 0.14	1.0 ± 0.02	1.1 ± 0.02	42.37
CDC Verano	32	17.98 ± 0.44	9.05 ± 0.09	1.9 ± 0.02	0.82 ± 0.13	1.05 ± 0.13	30.83
CDC Cory	16	37.36 ± 0.36	31.84 ± 0.6	2.37 ± 0.07	2.55 ± 0.12	2.04 ± 0.12	76.18
CDC Cory	24	22.36 ± 0.14	22. 16 ± 0.19	1.89 ± 0.0	1.43 ± 0.0	0.99 ± 0.01	48.84
CDC Cory	32	17.72 ± 0.18	18.51 ± 0.05	1.76 ± 0.14	0.86 ± 0.07	0.67 ± 0.07	39.54
CDC Jade	16	45.93 ± 0.64	26.12 ± 0.86	2.61 ± 0.06	2.88 ± 0.13	2.51 ± 0.13	80.07
CDC Jade	24	31.82 ± 0.14	17.89 ± 0.38	2.43 ± 0.02	2.54 ± 0.39	1.76 ± 0.39	56.44
CDC Jade	32	24.09 ± 0.34	14.76 ± 0.11	2.13 ± 0.01	1.74 ± 0.04	1.28 ± 0.04	44.03

5.3.4. Gene expression

One of the most key factors for real-time PCR data analysis is finding a good internal control since all the results would be normalized based on the cycle threshold (CT) of the house-keeping gene. The CT value for 18SrRNA was 14 with constant expression within and among the samples from five cultivars at different developmental stages, however, the CT value in other tested housekeeping genes was varied between 18 to 24 in different samples. Therefore, *18SrRNA* was used as internal control for relative quantitative expression analysis.

The expression of four genes *ZISO1*, *ZISO2*, *ZDS* and *VDE* were categorized in cluster I and the rest of carotenoid genes were included in cluster II (Figure 5.3). The expression level of phytoene synthase 1 was higher in early stage (8 DPA) than in the later stage in all cultivars. The highest and lowest transcript levels of phytoene synthase 1 were found in CDC Jade and CDC Frontier, respectively. The same pattern was observed for phytoene synthase 2 and 3. In contrast, phytoene synthase 4 had the lowest expression level in CDC Jade. The expression of phytoene desaturase

was dominant at 8 DPA. Its expression level was almost similar in all cultivars. Significant expression of carotene 15-*cis*-ζ-carotene isomerase 1 and 2 and ζ-carotene desaturase was obtained in CDC Jade and CDC 441-34 cultivars at 8 DPA (Figures 5.3, B2). The next two enzymes in the pathway, carotene isomerase 1 and 2, were expressed predominantly in kabuli type. Lycopene β-cyclase, lycopene ε-cyclase and carotenoid dioxygenase were highly expressed at 8 DPA in all cultivars. On average the lowest expression of β-carotene hydroxylase 1 and 2 was observed in CDC Jade and CDC Verano cultivars at 8 DPA. Expression of violaxanthin de-epoxidase was higher in CDC Jade at 8 DPA than other cultivars and the lowest expression was observed in CDC Frontier. Expression pattern of two zeaxanthin epoxidase was similar with higher expression at 8 DPA except for CDC Verano for which its expression increased at 16 DPA. The highest expression of neoxanthin synthase was observed in CDC 441-34 at 16 DPA (Figures 5.3, B2).

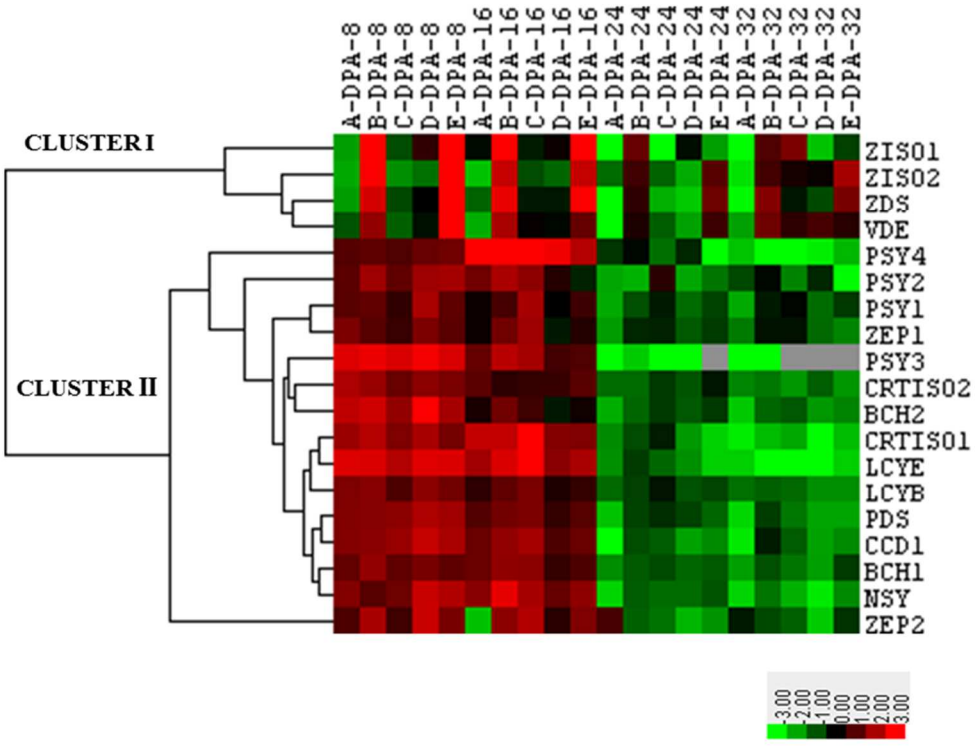


Figure 5.3. Heat map expression pattern of carotenogenic genes including Phytoene synthase 1 (*PSY1*), phytoene synthase 2 (*PSY2*), phytoene synthase 3 (*PSY3*), phytoene synthase 4 (*PSY4*), phytoene desaturase synthase (*PDS*), 15-cis-zeta-carotene isomerase 1 (*ZISO1*), 15-cis-zeta-carotene isomerase 1 (*ZISO2*), ζ-carotene desaturase (*ZDS*), carotene isomerase 1 (*CRTISO1*), carotene isomerase 2 (*CRTISO2*), lycopene β-cyclase (*LCYB*), lycopene ε-cyclase (*LCYE*), β-carotene hydroxylase 1 (*BCH1*), β-carotene hydroxylase 2 (*BCH2*), zeaxanthin epoxidase 1 (*ZEP1*) zeaxanthin epoxidase 2 (*ZEP2*), crotenoid 9,10(9',10')-cleavage dioxygenase 1 (*CCD1*) violaxanthin de-epoxidase

(*VDE*), and neoxanthin synthase (*NSY*) in chickpea seeds at four developmental stages 8, 16, 24 and 32 days post-anthesis (DPA) in five cultivars CDC Frontier (A), CDC 441-34 (B), CDC Verano (C), CDC Cory (D) and CDC Jade (E). Up-regulation and down-regulation were indicated in red and green respectively. No detection for expression with gray colour.

5.3.5. Correlation analysis

Correlation analysis revealed a positive correlation between expression of genes in the primary steps of carotenogenesis including zeta-carotene desaturase, zeta carotene isomerase and poly lycopene isomerase and carotenoid concentration. A negative correlation was obtained between hydroxylation and cleavage activities and provitamin A concentration (Table 5.3).

Table 5.3. Pearson correlation analysis between transcript levels of genes involved in the carotenoid pathway and their product in chickpea seed at different days post-anthesis (DPA). $p < 0.05$ and $p < 0.01$ were considered for significant (*) and highly significant (**), respectively.

Gene	Carotenoid type	Stage	Correlation
Zeta carotene isomerase 2	Lutein	32 DPA	0.90*
Zeta carotene isomerase 2	Zeaxanthin	32 DPA	0.86*
Zeta carotene isomerase 2	β-Carotene	32 DPA	0.94**
Zeta carotene isomerase 2	β-Cryptoxanthin	32 DPA	0.93**
Zeta carotene isomerase 2	Total carotenoids	32 DPA	0.90*
Neoxanthin synthase	Total carotenoids	8 DPA	0.87*

5.4. Discussion

The current study revealed that the majority of the SNPs within the genes involved in the carotenoid biosynthesis resulted from synonymous substitutions. Similar patterns of variation were also found in genes of carotenoid biosynthesis in tomato, citrus, pepper, and carrot (Livingstone and Anderson, 2009). The highly conserved carotenoid biosynthesis genes across different species reflect that these genes are required for a wide range of end products essential for the plants. Any changes on these enzymes could have major deleterious effects on plant fitness. Traditionally, researchers mainly focused on the non-synonymous mutations that can result in the changing of the amino acids and consequently protein function (Sauna and Kimchi-Sarfaty, 2011).

However, recent studies indicated that the synonymous mutations may have an effect on mRNA splicing (Pagani et al., 2005), mRNA stability (Kimchi-Sarfaty et al., 2007), the efficiency of protein translation, and protein folding (Sauna and Kimchi-Sarfaty, 2011). Protein expression

can also be influenced by synonymous SNPs as they are involved in regulating microRNA-mediated genes (Wang et al., 2015). Therefore, the synonymous SNPs identified in the diverse chickpea cultivars in this study may have potential functional significance in carotenoid biosynthesis. Further study might be needed to examine the detail of this mechanism.

The two non-synonymous SNPs in *ZISO2* in CDC Verano are good examples of mutations that changed the amino acids as a result. The enzyme *ZISO2* is involved in isomerization activities in the carotenoid pathway and its expression significantly correlated with various carotenoid concentrations. The green cotyledon kabuli, CDC Verano, had the highest carotenoid concentration among all the kabuli type which could be due to the mutations in *ZISO2* in this cultivar.

Variation of exon numbers among different copies of the carotenoid genes is interesting in this study. For example, among the different copies of the *CRTISO* gene the exon numbers varied from 5 to 13, while in *PSY* the exons varied from five to nine (Table A7). Exon-intron architecture is one of the mysterious issues in gene evolution (Zhu et al., 2009). It seems that the length and CG content of first exon and intron have an association with functional element in higher organism (Kalari et al., 2006). Kreimer and Pe'er, (2013) also discussed that exon variant can affect gene expression. It is possible that the changes in gene expression between different copy numbers of a carotenoid gene like *PSY* in chickpea might be affected by the variation in exon number and size. The 32 candidate genes involved in isoprenoid and carotenoid pathways were distributed across all 8 chromosomes of chickpea. Different copy numbers for some candidate genes exist in the chickpea genome (Figure 5.1). The *PSY* gene has been considered as a key regulator of the carotenoid pathway (Li et al., 2008). In domesticated maize, the *PSY1* locus has been the target of selective sweep and it was reported that 6.6–27.2% variations of the seed carotenoid concentration are associated with the activity of this enzyme (Bai et al., 2009; Chander et al., 2008; Palaisa et al., 2004). Pozniak et al. (2007) showed that *PSY1* is co-segregated with the 7B QTL in durum wheat (*Triticum turgidum* L. var *durum*) and confirmed the correlation of the gene with phenotypic variation for endosperm colour. Cereals commonly have three homologs of *PSY* in their genome. Study on *PSY* family in maize showed that the expression of each member can be different among various tissues. Usually, the expression of *PSY1* is higher in leaves and yellow endosperm, but *PSY2* expression is significant in almost all tissues (Gallagher et al., 2004). The third family member, *PSY3* is normally expressed in embryo and root especially under stress condition (Li et

al., 2008). In chickpea, we found four members of *PSY* family, which may have a positive effect on carotenoid concentration across different cotyledon colours.

Significant and positive correlation between isomerization activities and carotenoid concentration (Table 5.3) in chickpea indicated the key role of these enzymes in providing common precursor lycopene for different carotenoid types. We also found that desaturation reaction had a positive correlation (tend to be significant) with carotenoid concentration (data not shown).

In addition, the transcript levels of isoprenoid genes including 1-deoxy-D-xylulose-5-phosphate synthase (*DXS3*), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*DXR*), 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase (*HDR*), and geranylgeranyl diphosphate synthase (*GGPS1*; Julliard and Douce, 1991; Julliard, 1992; Lichtenthaler, 1999), can significantly affect carotenoid concentration in plant (Vallabhaneni and Wurtzel, 2009), as carotenoid biosynthesis is a derivative of isoprenoid pathway (Cuttriss et al., 2011). The deficiency of ζ-carotene desaturase (*ZDS*) gene in sunflower (*Helianthus annuus* L.) resulted in the accumulation of ζ-carotene and the absence of β-carotene, lutein and violaxanthin in cotyledon (Conti et al., 2004). In many cases, genetic transformation or overexpression of phytoene synthase and desaturase to crop plants has resulted in higher provitamin A and total carotenoid concentration (Ye et al., 2000; Paine et al., 2005; Diretto et al., 2007; Naqvi et al., 2009; Kim et al., 2012). This study showed that CDC Jade cultivar with highest carotenoid concentration also had the highest expression level of *ZDS*. The β-carotene hydroxylation converts provitamin A compounds into xanthophylls that have no provitamin A properties (Quinlan et al., 2012), so the challenge for breeders to develop cultivars with higher provitamin A concentration is limiting the β-carotene hydroxylation activities (Messias et al., 2014). Diretto et al. (2006) showed that silencing of β-carotene hydroxylase results in a higher β-carotene concentration in potato. In this study, we observed lower hydroxylation activities in CDC Verano and CDC Jade cultivars with high carotenoid concentration.

Cleavage activity is involved in apocarotenoid production that consequently reduces the carotenoid concentration (Auldridge et al., 2006). Also, the carotenoid cleavage dioxygenase has been considered as a negative regulator for β-carotene concentration in *Arabidopsis* and its expression had a direct effect on the degradation and turnover of carotenoids in seeds during maturity period (Gonzalez-Jorge et al., 2013; Rodriguez-Avila et al., 2011). For example, functional analysis of carotenoid cleavage dioxygenase mutant in *Arabidopsis* (*AtCCD1*), showed

higher carotenoid concentration in mature seeds of mutant cultivar than the wild type (Auldridge et al., 2006). The important effect of *ZEP1* region on carotenoid concentration in 281 maize lines with various kernel colours has been discussed by Owens et al. (2014). It seems that the ratio between carotenoid production and its conversion into apocarotenoid, play a key role in carotenoid concentration in chickpea. In cultivars, CDC Cory and CDC Jade with higher carotenoid concentration the conversion rate of carotenoids to apocarotenoids is lower than the other cultivars with low carotenoid levels. Based on our results the carotenoid levels decreased from earlier stage to later stage in chickpea seeds. The ABA concentration increases during seed development and it is positively correlated with embryo maturation (Wang et al., 1998). The legume seeds, which are mostly embryo, have a significant concentration of ABA (Goldberg et al., 1994). ABA is not the only cleavage product of carotenoids and there are more products like strigolactone (Booker et al., 2004). This process can explain the reduction of carotenoid during seed development. In addition, non-enzymatic activities including oxidative stress and lipid peroxidation involved in carotenoid degradation during seed desiccation (Mène-Saffrané et al., 2010; Sattler et al., 2004, 2006). However, our knowledge regarding regulatory mechanism responsible for carotenoid concentration and composition may not be complete as other potential mechanisms may occur (De Moura et al., 2015; Shumskaya and Wurtzel, 2013).

The two stay-green cultivars, CDC Jade and CDC Verano had the highest concentration of β-carotene among the desi and the kabuli types, respectively. Also, total carotenoid concentration is higher in pea and chickpea cultivars with green cotyledon colour compared to cultivars with yellow cotyledons (Ashokkumar et al., 2014), and the same results were obtained as the case of lutein in pea (Holasová et al., 2009). Mutation on the stay-green gene (*SGR*) that involves in degradation of chlorophyll resulted in delayed senescence and consequently green cotyledon chickpea seed. In thylakoid membranes, carotenoids are in a complex of chlorophyll and protein of Photosystem I and II. For example, the PSI contains high β-carotene concentration and PSII is rich in lutein. In addition, the high correlation was found between lutein and chlorophyll concentration in green pea (Holasovál et al., 2009). Importantly the increased levels of carotenoid products normally associated with both the chloroplastic and the cytosolic pathways, including mevalonate, sterols, and squalene, as well as triacyl- glycerides (Kumar et al., 2012). For instance, *PSY* has a coexpression with genes involve in the synthesis of plastoquinone, NAD(P)H dehydrogenase, tiorredoxin, plastocianin, and ferredoxin (Meier et al., 2011). It seems that

carotenoids in the complex of green tissues stay more stable than tissues with chlorophyll degradation. It is discussed by Ashokkumar et al. (2014) that higher lycopene cyclase activity is the main reason for higher total carotenoids in green cotyledon pea; however the cyclase activity in two green cotyledon chickpea was not higher than the other cultivars in this study. We believe that lower cleavage and hydroxylation activities have significant effects on carotenoid concentration in green chickpeas.

Desi chickpea usually has higher lutein concentration than the kabuli type likely due to higher grain weight in kabuli type (Ashokkumar et al., 2015). It seems that the chromosomal linkage and pleiotropy can address the association between higher lutein concentration and low seed weight (Abbo et al., 2005). The obtained results for carotenoid concentration in our work was consistent with results from earlier studies for kabuli and desi chickpeas (Ashokkumar et al., 2014, 2015). It was reported that carotenogenesis genes are active in photosynthetic organs under various light qualities and the levels of both chlorophyll and carotenoids will increase dramatically in de-etiolation period (Romer and Fraser, 2005; Toledo-Ortiz et al., 2010). In addition, the total concentration of xanthophylls in different plant species increases dramatically in a strong light condition that results in decreasing the ratio between "lutein (L) and the xanthophylls-cycle components, zeaxanthin, antheraxanthin, and violaxanthin (Z+A+V; Hirschberg, 2001)". In order to control the light effect on carotenoid types, we grew all plants in the greenhouse under controlled condition.

In conclusion, the structure and function of the genes in the carotenoid pathway are associated with the concentration of different carotenoid components in chickpea. New technology in genome sequencing has helped us to understand the details regarding the structure, polymorphism, copy number, and location of the genes involved in the carotenoid biosynthesis in chickpea seeds. Along with genome sequence data, the variability of the expression pattern and carotenoid concentration in five cultivars revealed a logical relationship between genotypes and phenotypes in this study. We demonstrated that synonymous mutations may have functional effects on the expression pattern of the different genes involved in the carotenoid biosynthesis pathway.

CHAPTER 6. GENERAL DISCUSSION, CONCLUSIONS, FUTURE RESEARCH AND APPLICATIONS

6.1. General discussion

Carotenoids with β ring such as β-carotene have the provitamin A properties which are converted to vitamin A in the human body (Stahl and Sies, 2005). Also, the nonprovitamin A carotenoids like lutein and zeaxanthin serve as antioxidants that reduce the risk of cancer, age-related macular degeneration (ARMD), and cardiovascular diseases (Fraser and Bramley, 2004). Deficiency of vitamin A has severe side effects on children like blindness, and night blindness in pregnant women which increases the risk of mortality (Fraser and Bramley, 2004). Investigation of the consequences of vitamin A deficiency in 40 countries since 1998 showed that 250,000–500,000 children with vitamin A deficiency were blinded and roughly, half of them died within 12 months due to eyesight loss (World Health Organization, 2009; Iannotti et al., 2013).

As the second most important pulse crop, chickpea is one of the alternative to meat in many third-world countries and contains significant levels of carotenoids among legumes (Abbo et al., 2005; Abu-Salem and Abou-Arab, 2011; Jukanti et al., 2012, Ashokkumar et al., 2014, 2015; Rezaei et al., 2016). Improvement of carotenoids in this crop helps to combat vitamin A deficiency in developing countries. The main reason for developing the Golden Rice (Ye et al., 2000) was that none of the rice cultivars, the major staple food in South and Southeast Asia, contained provitamin A. Significant variation for carotenoids/provitamin A among the chickpea accessions were observed in the current genotypic panel which corresponded to the cotyledon colour diversity (Ashokkumar et al., 2014, 2015; Rezaei et al., 2016). One way to improve carotenoid levels in crops is by using available natural variation for carotenoid/provitamin A through conventional breeding (Giuliano, 2017). Using molecular markers in combination with classical breeding will expedite the process of developing boosted vitamin A chickpeas. Marker-assisted breeding for carotenoid improvement has been successfully used in different crops such as wheat (Pozniak et al., 2007), sweet potato (Cervantes-Flores et al., 2011), and maize (Chandler et al., 2013).

The availability of reference genome in chickpea for both kabuli (Varshney et al., 2013a) and desi (Jain et al., 2013) opened opportunities for researchers to conduct detailed genomic studies on this crop. For example, the SNP array is one of the advanced methods for mapping and QTL studies with the high-throughput genotyping and is accessible to most breeders for

developing new cultivars (Roorkiwal et al., 2017). The SNP array has already been used in various types of experiments such as mapping studies and genomic selection (GA) for agronomically important traits, and assessment of genetic diversity and QTL analysis in different crops such as rice (Chen et al., 2014a; McCouch et al., 2010), maize (Ganal et al., 2011; Unterseer et al., 2014), sunflower (Bachlava et al., 2012), soybean (Song et al., 2013), oat (Tinker et al., 2014), cotton (Hulse-Kemp et al., 2015), wheat (Winfield et al., 2016), groundnut (Pandey et al., 2016), as well as chickpea (Roorkiwal et al., 2017). The genomic study of carotenoid properties in various crops has been conducted through both association studies (Owens et al., 2014; Suwarno et al., 2015; Esuma et al., 2016; Colasuonno et al., 2017; Rabbi et al., 2017) and linkage and QTL analysis (Abbo et al., 2005; Just et al., 2009; Zhang et al., 2009b; Blanco et al., 2011; Burt et al., 2011; Ana et al., 2013; Kandianis et al., 2013; Zhao et al., 2013; Ellison et al., 2017; Jittham et al., 2017).

The chickpea collection used in the first study contains accessions from 14 countries with a total of 172 accessions which vary in seed shape, seed coat, and cotyledon colours, yet only two major groups were identified within the collection using PCA and population structure analysis. This can be explained by the origination of kabuli from desi chickpea because of selection for low tannin content and white flower colour (Moreno and Cubero, 1978). This resulted in the narrow genetic diversity in chickpea, which is limiting chickpea improvement for various traits. Creating new populations from the most contrasting parents and deep genotyping for genome mapping and QTL studies should be beneficial for improvement of chickpea (Gaur et al., 2011; Jamalabadi et al., 2013). While both studies adopted the same genotyping and phenotyping approaches, the main difference between study I and II was the type of population. The GWAS successfully captured more genomic region associated with carotenoids compare to linkage QTL mapping in chickpea. In the association panel, the number of recombination events and genetic diversity was higher than in the F_2 populations. In a germplasm collection, the historical recombination events play a key role in the association study since they resulted in a better detection of crucial alleles which contribute to variation (Zhu et al., 2008a; Marwan et al., 2014), compared to the recent recombination events in F_2 populations. In linkage and QTL mapping, the SNPs that deviated from the 1:2:1 ratio were removed from the analysis.

CDC Jade, a green desi cultivar, was used as a common parent for developing the F_2 populations. Mutation on *SGR* gene in CDC Jade leads to green cotyledon colour (Varma Penmetsa, personal communication) and a higher carotenoid concentration than those with yellow

cotyledons (Rezaei et al., 2016). This mutation prevents the degradation of chlorophyll, stops the break down of photosystem that contains carotenoids, delays senescence, keeps the seed colour green and overall results in higher carotenoid levels in the plant (Barry et al., 2008; Holasovál et al., 2009; Ren et al., 2007a; Sato et al., 2007). In addition, two mutations in *ZISO2* in CDC Verano, a green kabuli cultivar, might explain the highest carotenoid concentration in CDC Verano among the kabuli cultivars (Rezaei et al., 2016). The positive association of the green cotyledon colour and the carotenoid concentration was observed in all three F_2 populations in which the progeny with green cotyledons showed higher carotenoid and provitamin A concentrations than the progeny with yellow cotyledon did. Moreover, the monogenic inheritance of green cotyledon colour facilitated its mapping in each F_2 population.

On average, the desi accessions had a higher carotenoid concentration than the kabuli type due to stronger pigmentation in both cotyledon and seed coats, which resulted in positive association with the carotenoid level in chickpeas (Ashokkumar et al., 2014). Other crops such as potatoes with yellow flesh had higher carotenoids than those with white flesh (Lu et al., 2001). For the two green chickpeas, CDC Verano green kabuli and CDC Jade green desi, the concentration of carotenoids in CDC Jade was higher than that of CDC Verano (Ashokkumar et al., 2015; Rezaei et al., 2016). This is due to dark green cotyledon colour in CDC Jade. The seed coat of the CDC Jade was also dark green whereas the seed coat colour of the CDC Verano was relatively transparent.

In the current research, the carotenoid profile and order of concentration were comparable in both chickpea diverse accessions and the three F_2 populations. Lutein was the dominant component followed by zeaxanthin and violaxanthin. The two provitamins A, β-carotene, and β-cryptoxanthin, were always high in green or orange cotyledon chickpeas. During the initial stages of seed development, when the seeds were relatively small and green, both provitamins A were detectable in all types of chickpeas (Rezaei et al., 2016). Carotenoid biosynthesis and its accumulation are controlled by sophisticated and complex biosynthesis pathways. The carotenoid pathway is fed by plastidic methylerythritol 4-phosphate (MEP) and cytosolic mevalonic acid (MVA) pathways (Eisenreich et al., 2001, 2004; Rodriguez-Concepcion and Boronat, 2002). Both pathways produce isopentenyl pyrophosphate (IPP) that is finally converted to geranylgeranyl pyrophosphate (GGPP) the precursor of carotenoid (Giuliano, 2014). In the GWAS, 1-deoxy-D-xylulose-5-phosphate synthase (*DXS*) enzyme (Estévez et al., 2001; Zhao et al., 2011), was

significantly associated with the provitamin A concentration. Interestingly, the gene pyruvate decarboxylase 1 (*PDC*), which is similar to *DXS* in terms of sequence and activity (Lange et al., 1998; Sprenger et al., 1997), was significantly associated with carotenoid components in both the diversity panel and the biparental mapping populations. Moreover, the genes from the cytochrome P450 family mostly involved in the hydroxylation of carotenoids in the pathway (Inoue, 2004), showed an association with different components in both studies. We expected to see the association between genes from the carotenoid pathway like phytoene synthase (*PSY*), the rate limiting enzyme in the carotenoid pathway (Rodriguez-Concepcion, 2010), with carotenoid components, but there was no association between *PSY* or other important genes from the carotenoid pathway with any of the components in our studies. Factors like insertions, deletions, copy number variations, translocations, and transversions make SNP-based genotyping less effective to capture all genomic variations (Saxena et al., 2014a; Wang et al., 2014b; Thudi et al., 2016). Thus, the expression of 19 key genes from the carotenoid pathway was analyzed over four seed developmental stages after flowering in chickpea. Variants associated with these genes were not detected in any mapping study. The levels of all carotenoids gradually decreased with maturation when seeds turn yellow, except for green chickpea, which showed higher expression of genes involved in oxidation and cleavage activities (Rezaei et al., 2016). Conversion of carotene to xanthophylls by hydroxylating enzymes (Quinlan et al., 2012) decreases the level of provitamin A and the cleavage activity diminishes the level of carotenoids by biosynthesis of apocarotenoids such as ABA and strigolactone from carotenoids (Goldberg et al., 1994; Booker et al., 2004). Two additional factors including oxidative stress and lipid peroxidation contribute to the degradation of carotenoids during seed maturity (Mène-Saffrané et al., 2010; Sattler et al., 2004, 2006). The carotenoid concentration decreased in green chickpeas during maturation but at a lower rate compared to yellow cotyledon chickpea. The highest expression of *PSY* was found in green chickpea CDC Jade, while the genes responsible for the hydroxylation of provitamin A showed a low expression in the two green cotyledon chickpeas, CDC Jade and CDC Verano. A lower hydroxylation activity along with higher *PSY* expression resulted in an elevated level of provitamin A and total carotenoids in CDC Jade. The results from the expression analysis combined with the QTL maping in study II confirmed the important association between green cotyledon colour and higher carotenoid levels. Other carotenoid genes such as 15-cis-zeta-carotene isomerase, ζ-carotene desaturase, lycopene β-cyclase, and lycopene ε-cyclase were not identified in any

mapping studies which could be due to a lack of the genome coverage by the SNP array used in genotyping. Nevertheless, the SNPs that showed highly significant association with carotenoids are potentially useful in the marker-assisted breeding for improving carotenoids in chickpea.

The chickpea-based products such as hummus, snack, roasted chickpeas and etc. in most of the world market are commonly derived from yellow cotyledon chickpeas. The acceptance of green chickpea for consumption might be difficult. In the case of maize, the acceptance of yellow maize was a challenge based on different studies (Pixley et al. 2013). Thus, to breed chickpeas for higher carotenoids/ provitamin A, factors like carotenoid hydroxylation, cleavage activities, cotyledon colour, environmental effects, and adaptation of people to chickpea with new product colour should be considered. Previous reports indicated that carotenoid in seeds was mostly affected by genotype, not by the environment (Abbo et al., 2010; Owens et al 2014). However, in our study, the effects of genotype, environment and genotype by environment interaction were significant on all carotenoid components, except for β-cryptoxanthin. The latter component was observed only in two accessions grown in Limerick, SK in 2016. This might explain the statistically non-significant effects of the genotype and environment on β-cryptoxanthin. Carotenoids and their derivatives are involved in various metabolic activities including plant response to photooxidative stress (Havaux, 2014) which may explain the differences in carotenoid levels between the two years in our first study. Different weather conditions such as the higher temperature in Limerick, SK (2016) in comparison to Elrose, SK in 2015 may explain the elevated carotenoid concentration in the chickpea panel. The high genetic variation in the chickpea panel combined with the contrasting locations and conditions were the main reasons for the significant effects of the environment on the carotenoid concentration.

6.2. Conclusions

Environment, genotype (cultivar) and genotype by environment interaction significantly affected the concentrations of most carotenoid components in chickpea. High-throughput genotyping in combination with high precision phenotyping resulted in the identification of extensive genetic factors associated with carotenoids across the diverse chickpea accessions. The GWAS showed that the carotenoid level is controlled by a moderate number of genomic loci, which are mostly involved in the isoprenoid, methylerythritol and carotenoid pathways, and apocarotenoid biosynthesis.

The cotyledon colour is strongly associated with the carotenoid/provitamin A concentration. Chickpeas with green cotyledon showed the highest level of carotenoids compared to the conventional yellow cotyledon chickpeas. The cotyledon colour, inherited as a single gene, was mapped on to the same genomic location in the three F_2 populations. The QTLs that contributed to the high phenotypic variation in different carotenoid components were identified in the three F_2 populations. Also, some of QTLs were conserved across the three populations. The pathway for carotenoid biosynthesis is complex with many genes involved that affect carotenoid levels in chickpea seeds. The expression of the genes involved in carotenoid biosynthesis as well as the concentration of carotenoid components including violaxanthin, lutein, zeaxanthin, β-carotene, and β-cryptoxanthin decreased during the maturation of the chickpea seeds. High cleavage activity, hydroxylation, and lipid oxidation along with the production of ABA (apo-carotenoid) are the main reasons for decreasing carotenoids during the seed maturity. In addition to mutation in *SGR* in CDC Verano, a green kabuli, two non-synonymous mutations were found in *ZISO2* which might positively affect the carotenoid level in this cultivar. The markers which tagged the QTLs for different carotenoid components in this research may have practical applications for selecting lines with favorable alleles through marker-assisted breeding approaches to improve carotenoid/provitamin A levels in chickpeas.

6.3. Future research and applications

Confirming the potential of candidate genes involved in the carotenoid and isoprenoid pathway, especially those that are not analyzed in this study using the available genetic approach, would be a leading objective in future research. A new member of *PSY* genes was identified for the first time in chickpea which can be tested for its functional analysis. The genes that provide a precursor for carotenoid biosynthesis as well as the hydroxylase and cleavage genes that diminish the level of provitamin A in plant are compelling candidates for further analysis. These genes are decisive targets and challenges for plant breeders working on carotenoid/provitamin A improvement.

Due to narrow genetic diversity in chickpea, expanding the germplasm base of the chickpea collection and its use for population development are necessary. Accessions with high carotenoid levels can be used in breeding programs to develop cultivars with improved carotenoid/provitamin A content. The chickpea accessions/breeding lines should be grown in wider environments to get

a better idea regarding the environmental effect on the carotenoid level. This will allow us to introduce materials to the appropriate environment to obtain the highest level of these components in chickpea. The green cotyledon colour is an interesting trait in chickpea that noticeably results in higher carotenoid concentration. Green chickpeas are the best candidate to improve carotenoids; nonetheless, since many traditional chickpea markets are unfamiliar with green cotyledon chickpea, future research should focus on people's acceptance of this product.

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APPENDIX A. SUPPLEMENTARY DATA

Table A1. List of 172 chickpea accessions with their name, country of origin, cotyledon colour, seed coat colour and 1000 seed weight which used in the first study.

Entry	Name	Class	Origin	Cotyledon colour	Seed coat colour	Seed weight
1	BS1-D-15	Desi	NA	Yellow	Brown 3	239.92
2	92117-25	Desi	Canada	Yellow	Brown 1	215.84
3	CDC Desiray	Desi	Canada	Yellow	Brown 1	157.12
4	CDC Frontier	Kabuli	Canada	Yellow	Transparent	279.87
5	1671-12	Kabuli	Canada	Yellow	Beige 1	338.74
6	CDC Vanguard	Desi	Canada	Yellow	Brown 2	178.06
7	1478-1	Kabuli	Canada	Yellow	Beige 1	285.14
8	418-59	Desi	Canada	Yellow	Brown 2	223.02
9	2384-6	Kabuli	Canada	Yellow	Beige 2	389.63
10	2401-4	Desi	Canada	Yellow	Brown 2	230.91
11	701-6	Kabuli	Canada	Yellow	Beige 1	281.44
12	1832-2	Desi	Canada	Yellow	Brown 2	257.38
13	1401-1	Kabuli	Canada	Yellow	Beige 1	279.72
14	FLIP88-85C	Kabuli	Syria	Yellow	Beige 1	262.49
15	820-24	Kabuli	Canada	Yellow	Beige 2	295.24
16	FLIP97-281C	Kabuli	Syria	Yellow	Beige 1	297.40
17	FLIP03-101C	Kabuli	Syria	Yellow	Beige 1	285.62
18	ILC 195	Kabuli	Russia	Yellow	Beige 1	234.59
19	463-2	Desi	Canada	Yellow	Brown 2	219.87
20	363T-13	Desi	Canada	Yellow	Brown 1	170.69
21	ILC5928	Desi	Morocco	Yellow	Brown 1	203.58
22	FLIP06-76C	Kabuli	Syria	Yellow	Beige 1	295.78
23	ILC 5913-1-2	Kabuli	Moldova	Yellow	Beige 1	213.45
24	ILC2506	Kabuli	Russia	Yellow	Beige 1	222.02
25	FLIP06-82C	Kabuli	Syria	Yellow	Beige 1	293.33
26	CDC Nika	Desi	Canada	Yellow	Brown 1	226.84
27	95NN-1	Kabuli	NA	Yellow	Beige 1	288.00
28	1649-2	Kabuli	Canada	Yellow	Beige 1	291.33
29	FLIP97-101C	Kabuli	Syria	Yellow	Beige 1	269.59
30	1672-20	Kabuli	Canada	Yellow	Beige 2	299.56
31	425-14	Desi	Canada	Yellow	Brown 2	208.67
32	1402-1	Kabuli	Canada	Yellow	Beige 1	283.33
33	494-4	Kabuli	Canada	Yellow	Beige 1	289.20
34	2343-18	Kabuli	Canada	Yellow	Beige 1	332.55
35	889-8	Kabuli	Canada	Yellow	Beige 1	267.11
36	1930-2	Kabuli	Canada	Yellow	Beige 2	288.01
37	CA05-75-16	Kabuli	NA	Yellow	Beige 1	279.78
38	FLIP84-188C	Kabuli	Syria	Yellow	Beige 1	224.14

39	AB06-160-4	Kabuli	NA	Yellow	Beige 1	323.28
40	FLIP90-96C	Kabuli	Syria	Yellow	Beige 1	280.14
41	AB06-64-2	Kabuli	NA	Yellow	Beige 2	254.77
42	1013-1	Desi	Canada	Yellow	Brown 1	225.39
43	ICC12004	Desi	Syria	Yellow	Brown 3	130.56
44	439as-22	Kabuli	Canada	Yellow	Beige 1	281.26
45	FLIP98-133C	Kabuli	Syria	Yellow	Beige 1	284.20
46	FLIP07-87C	Kabuli	Syria	Yellow	Beige 1	300.88
47	ICC4936	Desi	Greece	Yellow	Brown 1	229.92
48	ILC2956	Kabuli	USSR	Yellow	Beige 2	278.12
49	ILC3856	Kabuli	Morocco	Yellow	Beige 1	217.43
50	S95420	Kabuli	Canada	Yellow	Beige 1	265.82
51	DH27-4	Desi	NA	Yellow	Brown 1	165.73
52	FLIP97-45C	Kabuli	Syria	Yellow	Beige 1	302.80
53	1707-9	Kabuli	Canada	Yellow	Beige 1	321.06
54	438-29	Kabuli	Canada	Yellow	Beige 1	285.30
55	1735-5	Desi	Canada	Yellow	Brown 1	273.27
56	CDC Leader	Kabuli	Canada	Yellow	Beige 1	272.25
57	X04TH41-4	Kabuli	NA	Yellow	Beige 1	305.68
58	CDC Corinne	Desi	India	Yellow	Brown 2	215.52
59	2739-1	Kabuli	Canada	Yellow	Beige 1	316.42
60	1814-2	Desi	Canada	Yellow	Brown 2	171.54
61	AB06-156-2	Kabuli	NA	Yellow	Beige 1	354.99
62	Myles	Desi	USA	Yellow	Brown 1	143.37
63	AB06-159-2	Kabuli	NA	Yellow	Beige 1	319.76
64	897-14	Desi	Canada	Yellow	Brown 2	183.34
65	Amit	Kabuli	Canada	Yellow	Beige 1	207.40
66	316B-42	Desi	Canada	Yellow	Brown 1	160.84
67	294T-16	Desi	Canada	Yellow	Brown 1	176.70
68	FLIP05-145C	Kabuli	Syria	Yellow	Beige 1	313.81
69	441-34	Kabuli	Canada	Yellow	White-off	242.21
70	93-120-63	Kabuli	NA	Yellow	Beige 1	256.75
71	1701-1	Kabuli	Canada	Yellow	Beige 1	310.05
72	CDC Palmer (1041-3)	Kabuli	Canada	Yellow	Beige 2	340.98
73	1739-2	Desi	Canada	Yellow	Brown 1	267.64
74	ICC4475	Desi	India/ Iran	Orange	Black	175.12
75	FLIP86-6C	Kabuli	Syria	Yellow	Beige 1	275.48
76	2085-1	Kabuli	Canada	Yellow	Beige 1	317.77
77	FLIP93-93	Kabuli	Syria	Yellow	Beige 1	253.45
78	2144-1	Kabuli	Canada	Yellow	Beige 1	315.53
79	FLIP97-677C	Kabuli	Syria	Yellow	Beige 1	298.60
80	2770-1	Desi	Canada	Orange	Black	157.98
81	ILC 3279	Kabuli	Tunisia	Yellow	Beige 1	240.36
82	X05TH47-3	Kabuli	NA	Yellow	Beige 1	335.67

83	CDC Anna	Desi	Canada	Yellow	Brown 2	213.04
84	1569-1	Kabuli	Canada	Yellow	Beige 1	334.35
85	CDC Yuma	Kabuli	Canada	Yellow	Beige 1	263.22
86	846-1	Desi	Canada	Yellow	Brown 3	176.99
87	491-17	Kabuli	Canada	Yellow	Beige 1	243.15
88	356-6	Desi	Canada	Yellow	Brown 1	160.30
89	ICC14911	Kabuli	India	Yellow	Beige 2	176.32
90	FLIP05-43C	Kabuli	Syria	Yellow	Beige 1	312.44
91	ICC5124	Kabuli	Israel	Yellow	Beige 2	177.86
92	97-Indian2-107	Kabuli	NA	Yellow	Beige 1	258.45
93	553-1	Desi	Canada	Yellow	Brown 2	221.09
94	1647-8	Kabuli	Canada	Yellow	Beige 1	274.93
95	1044-6	Kabuli	Canada	Yellow	Beige 1	248.18
96	FLIP05-162C	Kabuli	Syria	Yellow	Beige 1	303.32
97	FLIP81-293C	Kabuli	Syria	Yellow	Beige 1	269.23
98	2072-2	Kabuli	Canada	Yellow	Beige 1	373.82
99	2012 CP-BC-2-437	Kabuli	NA	Yellow	Beige 2	331.08
100	1764-5	Kabuli	Canada	Yellow	Beige 1	279.24
101	Elixir	Kabuli	Portugal	Yellow	Beige 1	276.71
102	1349-1	Kabuli	Canada	Yellow	Beige 1	335.92
103	CDC Luna	Kabuli	Syria	Yellow	Beige 1	264.23
104	x2001th 75-4	Kabuli	NA	Yellow	Beige 1	297.34
105	CDC Jade	Desi	Canada	Green	Green	152.19
106	x2001th 76-15	Kabuli	NA	Yellow	Beige 1	250.76
107	FLIP98-135C	Kabuli	Syria	Yellow	Beige 2	274.71
108	CIABN-99PL27119	Kabuli	NA	Yellow	Beige 1	279.81
109	FLIP05-80C	Kabuli	Syria	Yellow	Beige 1	301.68
110	CDC Cabri	Desi	Canada	Yellow	Brown 1	253.15
111	ILC202	Kabuli	Russia	Yellow	Beige 1	253.52
112	FLIP95-48C	Kabuli	Syria	Yellow	Beige 1	349.20
113	CA05-73-6	Kabuli	NA	Yellow	Beige 1	230.74
114	95NN12	Kabuli	NA	Yellow	Beige 2	275.47
115	561aS-18	Kabuli	Canada	Yellow	Beige 1	230.88
116	1045-1	Kabuli	Canada	Yellow	Beige 1	263.30
117	1778-2	Kabuli	Canada	Yellow	Beige 2	306.30
118	FLIP82-150C	Kabuli	Syria	Yellow	Beige 1	225.96
119	FLIP07-40C	Kabuli	Syria	Yellow	Beige 1	330.66
120	2382-3	Kabuli	Canada	Yellow	Beige 1	314.58
121	FLIP93-58C	Kabuli	Syria	Yellow	Beige 1	240.26
122	2012 CP-BC-2-472	Kabuli	NA	Yellow	Beige 2	335.00
123	2223-2	Kabuli	Canada	Yellow	Beige 1	291.83
124	ILC 482	Kabuli	Turkey	Yellow	Beige 1	237.91
125	2293-3	Kabuli	Canada	Yellow	Beige 1	356.05
126	X05TH20-2	Kabuli	NA	Yellow	Beige 1	347.49

127	95168-64	Kabuli	Canada	Yellow	Beige 2	169.39
128	1136-3	Desi	Canada	Yellow	Brown 1	230.82
129	242-2	Kabuli	Canada	Yellow	Beige 1	266.39
130	548aS-20	Desi	Canada	Yellow	Beige 1	264.49
131	FLIP95-56C	Kabuli	Syria	Yellow	Beige 1	281.20
132	CDC Orion	Kabuli	Canada	Yellow	Beige 1	333.78
133	BS1-43	Desi	NA	Yellow	Brown 3	236.13
134	FLIP07-1C	Kabuli	Syria	Yellow	Beige 1	309.48
135	FLIP07-25C	Kabuli	Syria	Yellow	Beige 1	311.31
136	CDC Consul (603-3)	Desi	Canada	Yellow	Brown 3	258.65
137	GPE094	Desi	Canada	Yellow	Brown 2	240.46
138	1683-3	Kabuli	Canada	Yellow	Beige 2	300.85
139	1632-7	Kabuli	Canada	Yellow	Beige 2	318.92
140	FLIP87-45C	Kabuli	Syria	Yellow	Beige 1	274.63
141	2385-1	Kabuli	Canada	Yellow	Beige 2	364.05
142	FLIP97-137C	Kabuli	Syria	Yellow	Beige 1	294.31
143	2012 CP-BC-2-434	Kabuli	NA	Yellow	Beige 1	321.84
144	1757-8	Kabuli	Canada	Yellow	Beige 2	311.16
145	713-13	Kabuli	Canada	Yellow	Beige 2	330.98
146	2239-4	Kabuli	Canada	Yellow	Beige 2	306.40
147	CDC Chico	Kabuli	Canada	Yellow	Beige 1	195.49
148	AB06-106-2	Kabuli	NA	Yellow	Beige 1	353.29
149	95177-47	Kabuli	Canada	Yellow	Beige 1	215.46
150	1220-1	Desi	Canada	Yellow	Brown 1	248.63
151	97-Indian2-112	Kabuli	India	Yellow	Beige 1	304.88
152	x2001th 169-3	Kabuli	NA	Yellow	Beige 1	267.93
153	381T-4	Desi	Canada	Yellow	Brown 2	249.64
154	413-2	Desi	Canada	Yellow	Brown 2	219.42
155	492-24	Kabuli	Canada	Yellow	Beige 2	277.22
156	612-4	Desi	Canada	Yellow	Brown 2	262.47
157	1702-1	Kabuli	Canada	Yellow	Beige 2	301.11
158	FLIP84-92C	Kabuli	Syria	Yellow	Beige 1	280.21
159	1414-2	Kabuli	Canada	Yellow	Beige 2	301.71
160	2393-2	Kabuli	Canada	Yellow	Beige 2	342.84
161	FLIP 09-6C	Kabuli	Syria	Yellow	Beige 1	315.06
162	ILC 72	Kabuli	Spain	Yellow	Beige 1	233.13
163	1957-1	Kabuli	Canada	Yellow	Beige 1	271.44
164	1173-1	Kabuli	Canada	Yellow	Beige 2	325.82
165	FLIP05-46C	Kabuli	Syria	Yellow	Beige 1	289.33
166	CA05-75-45	Kabuli	NA	Yellow	Beige 1	277.51
167	FLIP06-116C	Kabuli	Syria	Yellow	Beige 1	332.13
168	1460-2	Desi	Canada	Yellow	Brown 1	190.63
169	FLIP07-39C	Kabuli	Syria	Yellow	Beige 1	292.38
170	x2001th 77-5	Kabuli	NA	Yellow	Beige 1	280.74

171	ICC1069	Black	USSR	Orange	Black	213.40
172	FLIP98-136C	Kabuli	Syria	Yellow	Beige 1	266.71

Note: The lower values for seed coat colour represent darker colour and vice versa

Table A2. Concentration (μg g^{-1}) of different seed carotenoid including violaxanthin, lutein, zeaxanthin, β-carotene, β-cryptoxanthin, and total carotenoids ± Se measured by HPLC in 172 chickpea accessions grown in Elrose, Saskatchewan in 2015.

Chickpea lines	Violaxanthin	Lutein	Zeaxanthin	β-Cryptoxanthin	β-carotene	Total
BS1-D-15	0.6 ± 0.28	11.74 ± 3.13	2.15 ± 0.65	0 ± 0	0 ± 0	14.49
92117-25	0.35 ± 0.1	14.53 ± 1.93	2.67 ± 0.51	0 ± 0	0.49 ± 0.49	18.04
CDC Desiray	0.13 ± 0.02	9.24 ± 3.37	2.09 ± 0.89	0 ± 0	0 ± 0	11.46
CDC Frontier	0.04 ± 0.04	8.03 ± 3.42	1.61 ± 0.76	0 ± 0	0 ± 0	9.68
1671-12	0.61 ± 0.21	9.48 ± 1.53	1.86 ± 0.43	0 ± 0	0 ± 0	11.95
CDC Vanguard	0.87 ± 0.09	5.57 ± 0.91	0.79 ± 0.2	0 ± 0	0 ± 0	7.23
1478-1	0.75 ± 0.27	8.12 ± 1.5	2.14 ± 0.47	0 ± 0	0 ± 0	11.01
418-59	0.53 ± 0.11	7.98 ± 3.11	1.35 ± 0.69	0 ± 0	0 ± 0	9.87
2384-6	0.91 ± 0.02	9.21 ± 2.73	2.1 ± 0.57	0 ± 0	0 ± 0	12.23
2401-4	0.39 ± 0.1	13.95 ± 0.63	2.61 ± 0.07	0 ± 0	0 ± 0	16.95
701-6	0.69 ± 0.03	5.32 ± 0.42	1.63 ± 0.22	0 ± 0	0 ± 0	7.63
1832-2	0.4 ± 0.13	14.45 ± 3.21	3.35 ± 0.7	0 ± 0	0 ± 0	18.20
1401-1	0.32 ± 0.1	10.67 ± 1.3	2.55 ± 0.45	0 ± 0	0 ± 0	13.54
FLIP88-85C	0.11 ± 0.07	6.67 ± 1.94	1.53 ± 0.41	0 ± 0	0 ± 0	8.31
820-24	0.08 ± 0.08	3.74 ± 0.34	1.13 ± 0.09	0 ± 0	0 ± 0	4.95
FLIP97-281C	0.18 ± 0.09	9.36 ± 2.57	2.46 ± 0.72	0 ± 0	0 ± 0	12.00
FLIP03-101C	0.32 ± 0.01	4.95 ± 0.7	1.29 ± 0.24	0 ± 0	0 ± 0	6.55
ILC 195	0.29 ± 0.15	4.79 ± 0.2	1.27 ± 0.04	0 ± 0	0 ± 0	6.35
463-2	0.06 ± 0.06	5.05 ± 1.28	0.98 ± 0.17	0 ± 0	0 ± 0	6.09
363T-13	0.29 ± 0.15	12.87 ± 1.5	3.05 ± 0.64	0 ± 0	0 ± 0	16.21
ILC5928	0.2 ± 0.1	10.43 ± 0.7	2.84 ± 0.14	0 ± 0	0 ± 0	13.47
FLIP06-76C	0.36 ± 0.15	7.09 ± 0.72	1.9 ± 0.28	0 ± 0	0 ± 0	9.35
ILC 5913-1-2	0.37 ± 0.15	18.75 ± 3.5	3.49 ± 0.41	0 ± 0	0.22 ± 0.22	22.82
ILC2506	0.26 ± 0.1	11.52 ± 2.72	3.26 ± 0.75	0 ± 0	0 ± 0	15.04
FLIP06-82C	0.02 ± 0.02	4.58 ± 0.53	1.1 ± 0.13	0 ± 0	0 ± 0	5.70
CDC Nika	0.72 ± 0.2	11.56 ± 1.77	2.74 ± 0.44	0 ± 0	0 ± 0	15.02
95NN-1	0.56 ± 0.07	11.45 ± 1.43	2.24 ± 0.18	0 ± 0	0 ± 0	14.24
1649-2	0.36 ± 0.01	7.22 ± 0.82	1.47 ± 0.16	0 ± 0	0 ± 0	9.05
FLIP97-101C	0.09 ± 0.04	8.96 ± 0.9	1.9 ± 0.2	0 ± 0	0 ± 0	10.95
1672-20	0.9 ± 0.05	8.1 ± 1.46	1.81 ± 0.36	0 ± 0	0 ± 0	10.81
425-14	0.14 ± 0.14	6.2 ± 3.59	0.99 ± 0.72	0 ± 0	0 ± 0	7.33
1402-1	0.82 ± 0.02	7.51 ± 0.53	1.96 ± 0.24	0 ± 0	0 ± 0	10.29
494-4	0.58 ± 0.07	6.34 ± 0.18	1.78 ± 0.07	0 ± 0	0 ± 0	8.71
2343-18	0.44 ± 0.08	9.7 ± 2.06	2.48 ± 0.53	0 ± 0	0 ± 0	12.62
889-8	0.16 ± 0.06	4.82 ± 0.88	1.25 ± 0.19	0 ± 0	0 ± 0	6.22
1930-2	0.5 ± 0.02	6.2 ± 2.03	1.48 ± 0.39	0 ± 0	0 ± 0	8.18

CA05-75-16	0.43 ± 0.06	7.8 ± 2.65	1.8 ± 0.38	0 ± 0	0 ± 0	10.03
FLIP84-188C	0.29 ± 0.05	9.77 ± 1.46	2.5 ± 0.36	0 ± 0	0 ± 0	12.56
AB06-160-4	0.14 ± 0.14	10.33 ± 1.6	2.56 ± 0.51	0 ± 0	0 ± 0	13.02
FLIP90-96C	0.44 ± 0.06	11.09 ± 0.53	2.68 ± 0.17	0 ± 0	0 ± 0	14.21
AB06-64-2	0.15 ± 0.09	4.42 ± 1.2	1.21 ± 0.3	0 ± 0	0 ± 0	5.78
1013-1	0.52 ± 0.29	8.62 ± 4.79	1.58 ± 0.44	0 ± 0	0 ± 0	10.72
ICC12004	0.55 ± 0.12	4.02 ± 0.65	0.39 ± 0.21	0 ± 0	0 ± 0	4.96
439as-22	0.06 ± 0.03	11.05 ± 1.93	2.66 ± 0.48	0 ± 0	0 ± 0	13.77
FLIP98-133C	0.21 ± 0.16	4.5 ± 1.85	0.81 ± 0.47	0 ± 0	0 ± 0	5.53
FLIP07-87C	0.19 ± 0.01	10.42 ± 0.99	2.73 ± 0.22	0 ± 0	0 ± 0	13.34
ICC4936	0.42 ± 0.08	11.7 ± 0.47	2.71 ± 0.17	0 ± 0	0 ± 0	14.83
ILC2956	0.25 ± 0.06	9.82 ± 2.14	2.8 ± 0.67	0 ± 0	0 ± 0	12.87
ILC3856	0.37 ± 0.08	14.25 ± 1.53	4.17 ± 0.51	0 ± 0	0 ± 0	18.80
S95420	0.04 ± 0.04	9.62 ± 1.38	2.2 ± 0.32	0 ± 0	0 ± 0	11.86
DH27-4	0.32 ± 0.03	18.19 ± 5.66	2.77 ± 1.19	0 ± 0	0 ± 0	21.29
FLIP97-45C	0.03 ± 0.03	6.14 ± 0.34	1.3 ± 0.13	0 ± 0	0 ± 0	7.47
1707-9	0.4 ± 0	7.37 ± 0.72	1.53 ± 0.15	0 ± 0	0 ± 0	9.30
438-29	0.1 ± 0.1	11.01 ± 0.89	1.88 ± 0.21	0 ± 0	0.26 ± 0.26	13.26
1735-5	0.45 ± 0.18	7.88 ± 0.31	1.5 ± 0.13	0 ± 0	0 ± 0	9.83
CDC Leader	0.78 ± 0.06	12.15 ± 1.24	2.94 ± 0.2	0 ± 0	0 ± 0	15.87
X04TH41-4	0.86 ± 0.02	8.06 ± 2.35	1.95 ± 0.6	0 ± 0	0 ± 0	10.86
CDC Corinne	0.57 ± 0.12	11.81 ± 3.29	2.29 ± 0.66	0 ± 0	0.2 ± 0.2	14.87
2739-1	0.35 ± 0.01	7.84 ± 0.78	1.78 ± 0.14	0 ± 0	0 ± 0	9.97
1814-2	0.18 ± 0.05	6.21 ± 1.09	1.49 ± 0.19	0 ± 0	0 ± 0	7.88
AB06-156-2	0.29 ± 0.06	10.96 ± 1.94	2.73 ± 0.55	0 ± 0	0 ± 0	13.98
Myles	0.92 ± 0.19	10.52 ± 3.8	1.75 ± 0.67	0 ± 0	0 ± 0	13.19
AB06-159-2	0.2 ± 0.06	4.25 ± 1.71	1.25 ± 0.48	0 ± 0	0 ± 0	5.70
897-14	0.33 ± 0.06	3.71 ± 0.63	0.75 ± 0.11	0 ± 0	0 ± 0	4.79
Amit	0.04 ± 0.04	11.69 ± 0.9	2.91 ± 0.31	0 ± 0	0 ± 0	14.64
316B-42	1.29 ± 0.26	9.56 ± 0.21	2.32 ± 0.06	0 ± 0	0 ± 0	13.17
294T-16	0.32 ± 0.09	8.17 ± 1.59	1.57 ± 0.45	0 ± 0	0 ± 0	10.06
FLIP05-145C	0.42 ± 0.16	6.33 ± 1.91	1.82 ± 0.55	0 ± 0	0 ± 0	8.57
441-34	0.47 ± 0.02	8.83 ± 0.76	2.27 ± 0.27	0 ± 0	0 ± 0	11.57
93-120-63	0.45 ± 0.02	7.95 ± 2.2	2.27 ± 0.74	0 ± 0	0 ± 0	10.67
1701-1	0.58 ± 0.05	7.34 ± 0.81	1.47 ± 0.22	0 ± 0	0 ± 0	9.39
CDC Palmer (1041-3)	0.26 ± 0.04	6.99 ± 1.6	1.74 ± 0.35	0 ± 0	0 ± 0	8.98
1739-2	0.96 ± 0.03	10.3 ± 1.35	2.17 ± 0.35	0 ± 0	0 ± 0	13.43
ICC4475	0.67 ± 0.09	12.68 ± 4.76	1.3 ± 0.53	0 ± 0	0 ± 0	14.64
FLIP86-6C	0.38 ± 0.04	8.32 ± 2.14	2.04 ± 0.46	0 ± 0	0 ± 0	10.75
2085-1	0.29 ± 0.08	8.96 ± 1.63	2.44 ± 0.42	0 ± 0	0 ± 0	11.69
FLIP93-93	0.39 ± 0.06	10.48 ± 3.43	2.55 ± 0.79	0 ± 0	0 ± 0	13.41
2144-1	0.37 ± 0.14	10.67 ± 2.45	2.84 ± 0.76	0 ± 0	0 ± 0	13.89
FLIP97-677C	0.26 ± 0.09	10.51 ± 3.11	2.48 ± 0.76	0 ± 0	0 ± 0	13.25
2770-1	0.96 ± 0.14	13.39 ± 5.19	1.2 ± 0.43	0 ± 0	0 ± 0	15.55

ILC 3279	0.08 ± 0.08	14.18 ± 1.97	3.69 ± 0.44	0 ± 0	0 ± 0	17.95
X05TH47-3	0.24 ± 0.06	9.12 ± 1.02	2.32 ± 0.29	0 ± 0	0 ± 0	11.69
CDC Anna	0.75 ± 0.21	14 ± 4.39	3.09 ± 1.23	0 ± 0	0 ± 0	17.83
1569-1	0.27 ± 0.04	4.45 ± 0.9	1.21 ± 0.21	0 ± 0	0 ± 0	5.93
CDC Yuma	0.2 ± 0.2	7.76 ± 2.97	1.9 ± 0.98	0 ± 0	0 ± 0	9.86
846-1	0.26 ± 0.02	2.09 ± 0.13	0.49 ± 0.13	0 ± 0	0 ± 0	2.84
491-17	0.04 ± 0.04	9.89 ± 1.44	2.09 ± 0.26	0 ± 0	0 ± 0	12.02
356-6	0.33 ± 0.04	8.01 ± 1.69	1.43 ± 0.25	0 ± 0	0 ± 0	9.76
ICC14911	0.34 ± 0.1	13.18 ± 4.45	3.27 ± 1.13	0 ± 0	0 ± 0	16.79
FLIP05-43C	0.57 ± 0.12	7.55 ± 0.34	2.24 ± 0.18	0 ± 0	0 ± 0	10.36
ICC5124	0 ± 0	4.09 ± 0.3	1.19 ± 0.1	0 ± 0	0 ± 0	5.28
97-Indian2-107	0.32 ± 0.02	4.91 ± 1.08	1.18 ± 0.25	0 ± 0	0 ± 0	6.42
553-1	1.08 ± 0.05	11.79 ± 1.43	2.62 ± 0.35	0 ± 0	0 ± 0	15.49
1647-8	0.43 ± 0.2	10.33 ± 1.6	2.14 ± 0.39	0 ± 0	0 ± 0	12.89
1044-6	0.51 ± 0.06	11.63 ± 0.98	2.76 ± 0.22	0 ± 0	0 ± 0	14.91
FLIP05-162C	0.88 ± 0.03	7.6 ± 1	1.77 ± 0.26	0 ± 0	0 ± 0	10.26
FLIP81-293C	0.37 ± 0.08	11.85 ± 1.4	2.81 ± 0.26	0 ± 0	0 ± 0	15.03
2072-2	0.87 ± 0	9.45 ± 3.19	2.41 ± 0.85	0 ± 0	0 ± 0	12.73
2012 CP-BC-2-437	0.4 ± 0.08	10.91 ± 1.69	2.56 ± 0.32	0 ± 0	0 ± 0	13.88
1764-5	0.23 ± 0.07	7.29 ± 0.48	1.87 ± 0.16	0 ± 0	0 ± 0	9.40
Elixir	0.32 ± 0.28	11.34 ± 3.28	2.59 ± 0.65	0 ± 0	0 ± 0	14.25
1349-1	0.06 ± 0.06	4.31 ± 1.14	1.85 ± 0.19	0 ± 0	0 ± 0	6.22
CDC Luna	0.44 ± 0.3	10.6 ± 0.59	2.1 ± 0.1	0 ± 0	0 ± 0	13.13
x2001th 75-4	0.37 ± 0.01	4.92 ± 0.93	1.46 ± 0.22	0 ± 0	0 ± 0	6.74
CDC Jade	3.06 ± 0.13	20.49 ± 0.72	1.92 ± 0.07	0 ± 0	2.19 ± 0.04	27.66
x2001th 76-15	0 ± 0	2.92 ± 0.13	0.82 ± 0.07	0 ± 0	0 ± 0	3.74
FLIP98-135C	0.49 ± 0.03	5.29 ± 2.05	1.3 ± 0.44	0 ± 0	0 ± 0	7.08
CIABN-99PL27119	0.19 ± 0.19	7.88 ± 0.6	2 ± 0.22	0 ± 0	0 ± 0	10.07
FLIP05-80C	0.19 ± 0.11	5.22 ± 1.04	1.37 ± 0.31	0 ± 0	0 ± 0	6.78
CDC Cabri	0.75 ± 0.02	9.31 ± 1.63	1.72 ± 0.46	0 ± 0	0 ± 0	11.78
ILC202	0.17 ± 0.02	12.05 ± 1.31	3.32 ± 0.42	0 ± 0	0 ± 0	15.54
FLIP95-48C	0.04 ± 0.04	8.11 ± 0.92	1.7 ± 0.19	0 ± 0	0 ± 0	9.85
CA05-73-6	0.54 ± 0.08	10.01 ± 5.23	2.26 ± 0.91	0 ± 0	0 ± 0	12.81
95NN12	0.75 ± 0.19	12.12 ± 0.07	2.92 ± 0.11	0 ± 0	0 ± 0	15.79
561aS-18	0.71 ± 0.02	8.9 ± 1.13	2.46 ± 0.32	0 ± 0	0 ± 0	12.08
1045-1	0.44 ± 0.01	11.81 ± 1.85	2.47 ± 0.26	0 ± 0	0.98 ± 0.08	15.70
1778-2	0.57 ± 0.22	6.06 ± 0.61	1.3 ± 0.07	0 ± 0	0 ± 0	7.94
FLIP82-150C	0.37 ± 0.06	8.19 ± 2.46	2.06 ± 0.59	0 ± 0	0 ± 0	10.62
FLIP07-40C	0.92 ± 0.02	8.91 ± 1.32	1.99 ± 0.3	0 ± 0	0 ± 0	11.82
2382-3	0.91 ± 0.04	9.96 ± 0.23	2.28 ± 0.07	0 ± 0	0 ± 0	13.15
FLIP93-58C	0.26 ± 0.05	7.04 ± 2.5	1.98 ± 0.72	0 ± 0	0 ± 0	9.28
2012 CP-BC-2-472	0.49 ± 0.13	9.69 ± 1.73	2.38 ± 0.37	0 ± 0	0 ± 0	12.55
2223-2	0.32 ± 0.12	10.52 ± 2.32	2.73 ± 0.58	0 ± 0	0 ± 0	13.57
ILC482	0 ± 0	6.71 ± 1.48	1.56 ± 0.36	0 ± 0	0 ± 0	8.27

2293-3	0.29 ± 0.02	10.67 ± 1.19	2.71 ± 0.37	0 ± 0	0 ± 0	13.67
X05TH20-2	0.4 ± 0.03	9.39 ± 2.61	2.38 ± 0.7	0 ± 0	0 ± 0	12.18
95168-64	0 ± 0	5.93 ± 1.95	1.16 ± 0.44	0 ± 0	0 ± 0	7.09
1136-3	0.33 ± 0.03	6.78 ± 1.44	1.78 ± 0.26	0 ± 0	0 ± 0	8.89
242-2	0.03 ± 0.03	7.49 ± 1.13	1.57 ± 0.3	0 ± 0	0 ± 0	9.09
548aS-20	0.27 ± 0.08	4.73 ± 2.66	0.66 ± 0.2	0 ± 0	0.16 ± 0.16	5.82
FLIP95-56C	0.38 ± 0.14	7.02 ± 1.49	1.61 ± 0.33	0 ± 0	0 ± 0	9.00
CDC Orion	0.66 ± 0.03	6.13 ± 1.83	1.58 ± 0.42	0 ± 0	0 ± 0	8.37
BS1-43	0.55 ± 0.16	16.97 ± 3.44	3.17 ± 0.8	0 ± 0	0.97 ± 0.49	21.66
FLIP07-1C	0.22 ± 0.19	12.34 ± 0.9	3.04 ± 0.36	0 ± 0	0 ± 0	15.61
FLIP07-25C	0.33 ± 0.03	16 ± 2.87	3.27 ± 0.19	0 ± 0	0 ± 0	19.60
CDC Consul (603-3)	1 ± 0.07	11.45 ± 1.64	2.69 ± 0.33	0 ± 0	0 ± 0	15.14
GPE094	0.45 ± 0.05	7.22 ± 0.61	1.51 ± 0.13	0 ± 0	0 ± 0	9.18
1683-3	0.59 ± 0.18	5.79 ± 1.17	1.38 ± 0.24	0 ± 0	0 ± 0	7.76
1632-7	0.98 ± 0.01	9.23 ± 0.81	2.04 ± 0.07	0 ± 0	0 ± 0	12.25
FLIP87-45C	0.27 ± 0.03	10.47 ± 2.85	2.66 ± 0.75	0 ± 0	0 ± 0	13.40
2385-1	1 ± 0.02	8.02 ± 1.11	1.83 ± 0.12	0 ± 0	0 ± 0	10.85
FLIP97-137C	0.65 ± 0.09	11.93 ± 2.74	2.79 ± 0.7	0 ± 0	0 ± 0	15.37
2012 CP-BC-2-434	0.33 ± 0.12	10.87 ± 3.82	2.68 ± 0.89	0 ± 0	0 ± 0	13.88
1757-8	0.2 ± 0.08	8.5 ± 0.92	2.15 ± 0.23	0 ± 0	0 ± 0	10.85
713-13	0 ± 0	10.46 ± 2.54	2.41 ± 0.62	0 ± 0	0 ± 0	12.87
2239-4	0.26 ± 0.1	6.88 ± 1.36	1.66 ± 0.27	0 ± 0	0 ± 0	8.80
CDC Chico	0.04 ± 0.04	7.74 ± 2.91	1.64 ± 0.67	0 ± 0	0 ± 0	9.43
AB06-106-2	0.05 ± 0.05	3.21 ± 0.3	0.55 ± 0.23	0 ± 0	0 ± 0	3.81
95177-47	0.05 ± 0.05	9.57 ± 0.96	2.23 ± 0.37	0 ± 0	0 ± 0	11.85
1220-1	0.41 ± 0.03	4.78 ± 2	1.39 ± 0.49	0 ± 0	0 ± 0	6.57
97-Indian2-112	0.18 ± 0.14	9.41 ± 2.35	2.25 ± 0.63	0 ± 0	0 ± 0	11.85
x2001th 169-3	0.15 ± 0.02	5.4 ± 0.71	1.28 ± 0.21	0 ± 0	0 ± 0	6.82
381T-4	0.55 ± 0.07	8.01 ± 0.63	1.61 ± 0.07	0 ± 0	0 ± 0	10.17
413-2	0 ± 0	7.62 ± 1.45	1.27 ± 0.11	0 ± 0	0 ± 0	8.89
492-24	0.57 ± 0.02	5.8 ± 1.64	1.56 ± 0.3	0 ± 0	0 ± 0	7.92
612-4	0.92 ± 0.06	13.6 ± 2.98	3.36 ± 0.65	0 ± 0	0 ± 0	17.89
1702-1	0.58 ± 0.29	4.97 ± 2.5	0.95 ± 0.49	0 ± 0	0 ± 0	6.50
FLIP84-92C	0.31 ± 0.08	12.9 ± 3.18	3.06 ± 0.68	0 ± 0	0 ± 0	16.27
1414-2	0.79 ± 0.04	8.16 ± 0.62	2.25 ± 0.14	0 ± 0	0 ± 0	11.19
2393-2	0.77 ± 0.11	6.68 ± 0.24	1.51 ± 0.15	0 ± 0	0 ± 0	8.96
FLIP 09-6C	0.44 ± 0.06	11.12 ± 1.83	2.33 ± 0.17	0 ± 0	0 ± 0	13.89
ILC 72	0.27 ± 0.14	7.9 ± 1.76	2.05 ± 0.57	0 ± 0	0 ± 0	10.22
1957-1	0.11 ± 0.07	4.66 ± 1.63	1.21 ± 0.41	0 ± 0	0 ± 0	5.98
1173-1	0.05 ± 0.05	6.35 ± 1.54	1.51 ± 0.43	0 ± 0	0 ± 0	7.91
FLIP05-46C	0.23 ± 0.06	10.09 ± 1.32	2.52 ± 0.22	0 ± 0	0 ± 0	12.85
CA05-75-45	0.06 ± 0.03	5.46 ± 0.2	1.31 ± 0.07	0 ± 0	0 ± 0	6.83
FLIP06-116C	0.12 ± 0.06	8.31 ± 2.04	2.21 ± 0.51	0 ± 0	0 ± 0	10.64
1460-2	0 ± 0	2.05 ± 0.14	0.55 ± 0.03	0 ± 0	0 ± 0	2.60

FLIP07-39C	0.3 ± 0.13	10.54 ± 1.16	2.72 ± 0.25	0 ± 0	0 ± 0	13.56
x2001th 77-5	0.26 ± 0.03	3.45 ± 0.38	1 ± 0.1	0 ± 0	0 ± 0	4.71
ICC1069	0.74 ± 0.14	21.04 ± 0.91	2.29 ± 0.29	0 ± 0	0 ± 0	24.07
FLIP98-136C	0.14 ± 0.01	8.17 ± 0.99	1.95 ± 0.23	0 ± 0	0 ± 0	10.26

Table A3. Concentration (µg g^{-1}) of different seed carotenoid including violaxanthin, lutein, zeaxanthin, β-carotene, β-cryptoxanthin, and total carotenoids ± Se measured by HPLC in 172 chickpea accessions grown in Limerick, Saskatchewan in 2016.

Chickpea lines	Violaxanthin	Lutein	Zeaxanthin	β-Cryptoxanthin	β-carotene	Total
BS1-D-15	0.61 ± 0.27	22.47 ± 2.16	2.27 ± 0.24	0 ± 0	0.98 ± 0.49	26.33
92117-25	0.37 ± 0.15	16.59 ± 4.86	0.73 ± 0.73	0 ± 0	0.36 ± 0.36	18.05
CDC Desiray	0.06 ± 0.03	9.46 ± 5.86	0.01 ± 0	0 ± 0	0 ± 0	9.52
CDC Frontier	0.15 ± 0.03	15.54 ± 2.25	1.88 ± 0.23	0 ± 0	0 ± 0	17.57
1671-12	0.37 ± 0.03	13.42 ± 1.86	2.13 ± 0.33	0 ± 0	0 ± 0	15.93
CDC Vanguard	0.48 ± 0.21	10.3 ± 0.89	1.01 ± 0.51	0 ± 0	0.4 ± 0.4	12.20
1478-1	1.28 ± 0.76	18.53 ± 1.46	2.69 ± 0.26	0 ± 0	0.7 ± 0.7	23.21
418-59	0.64 ± 0.06	11.25 ± 1.69	1.27 ± 0.2	0 ± 0	0 ± 0	13.17
2384-6	0.31 ± 0.06	7.1 ± 1.58	1.06 ± 0.39	0 ± 0	0 ± 0	8.47
2401-4	0.41 ± 0.13	8.75 ± 3.73	1.14 ± 0.61	0 ± 0	0 ± 0	10.30
701-6	0.22 ± 0.05	5.55 ± 0.79	0.15 ± 0.08	0 ± 0	0 ± 0	5.92
1832-2	0.45 ± 0.06	15.71 ± 3.63	2.56 ± 0.51	0 ± 0	0 ± 0	18.72
1401-1	0.08 ± 0.01	8.58 ± 3.38	0.01 ± 0	0 ± 0	0.48 ± 0.48	9.15
FLIP88-85C	0.4 ± 0.07	4.15 ± 1.68	0.01 ± 0	0 ± 0	0 ± 0	4.56
820-24	0.05 ± 0.05	9.39 ± 4.05	0.01 ± 0	0 ± 0	0.35 ± 0.35	9.79
FLIP97-281C	0.07 ± 0.01	13.48 ± 5.68	0.7 ± 0.69	0 ± 0	0 ± 0	14.25
FLIP03-101C	0.28 ± 0.01	14.98 ± 0.55	1.89 ± 0.18	0 ± 0	0.41 ± 0.41	17.56
ILC 195	0.07 ± 0.01	11.78 ± 1.04	0.65 ± 0.33	0 ± 0	0 ± 0	12.49
463-2	0.08 ± 0.01	2.2 ± 0.62	0.01 ± 0	0 ± 0	0 ± 0	2.29
363T-13	0.69 ± 0.18	14.4 ± 1.81	0.01 ± 0	0 ± 0	0 ± 0	15.10
ILC5928	0.5 ± 0.08	12.34 ± 1.43	2.1 ± 0.24	0 ± 0	0 ± 0	14.94
FLIP06-76C	0.31 ± 0.05	7.49 ± 2.37	1.19 ± 0.52	0 ± 0	0 ± 0	8.99
ILC 5913-1-2	0.1 ± 0.02	10.54 ± 1.67	2 ± 0.38	0 ± 0	0 ± 0	12.64
ILC2506	0.27 ± 0.18	10.31 ± 0.52	1.8 ± 0.14	0 ± 0	0 ± 0	12.37
FLIP06-82C	0.27 ± 0.11	10.99 ± 1.12	1.71 ± 0.28	0 ± 0	0 ± 0	12.97
CDC Nika	0.15 ± 0.09	13.29 ± 4.4	0.01 ± 0	0 ± 0	0 ± 0	13.45
95NN-1	0.11 ± 0.02	9.98 ± 1.13	0.57 ± 0.57	0 ± 0	0 ± 0	10.66
1649-2	0.41 ± 0.26	10.77 ± 3.41	1.17 ± 0.56	0 ± 0	0 ± 0	12.36
FLIP97-101C	0.36 ± 0.12	11.59 ± 1.31	1.81 ± 0.2	0 ± 0	0 ± 0	13.76
1672-20	0.08 ± 0.08	13.11 ± 1.77	2.3 ± 0.29	0 ± 0	0 ± 0	15.49
425-14	0.87 ± 0.41	10.85 ± 4.39	0.75 ± 0.74	0.42 ± 0.42	0.79 ± 0.79	13.68
1402-1	0.61 ± 0.28	19.3 ± 4.36	3.78 ± 1.06	0 ± 0	1.73 ± 0.39	25.42
494-4	0.44 ± 0.17	8.28 ± 4.11	0.71 ± 0.59	0 ± 0	0 ± 0	9.43

2343-18	0.48 ± 0.09	12.71 ± 5.54	12.77 ± 8.64	0 ± 0	0.45 ± 0.45	26.41
889-8	0.31 ± 0.12	8.69 ± 1.48	0.96 ± 0.39	0 ± 0	0 ± 0	9.96
1930-2	0.28 ± 0.07	7.6 ± 1.84	1.35 ± 0.42	0 ± 0	0 ± 0	9.23
CA05-75-16	0.43 ± 0.11	3.39 ± 1.47	0.38 ± 0.38	0 ± 0	0 ± 0	4.21
FLIP84-188C	0.28 ± 0.08	8.13 ± 4.3	0.01 ± 0	0 ± 0	0 ± 0	8.41
AB06-160-4	0.08 ± 0.02	15.39 ± 2.28	0.01 ± 0	0 ± 0	1.48 ± 0.41	16.96
FLIP90-96C	0.42 ± 0.08	6.45 ± 0.4	0.01 ± 0	0 ± 0	0 ± 0	6.88
AB06-64-2	0.05 ± 0.03	13.53 ± 1.18	0.01 ± 0	0 ± 0	0 ± 0	13.59
1013-1	0.41 ± 0.18	9.34 ± 2.07	0.34 ± 0.33	0 ± 0	0 ± 0	10.09
ICC12004	0.98 ± 0.64	11.66 ± 4.49	0.92 ± 0.47	0 ± 0	1.16 ± 1.16	14.71
439as-22	0.21 ± 0.02	15.11 ± 6.13	1.06 ± 1.05	0 ± 0	0.59 ± 0.3	16.98
FLIP98-133C	0.37 ± 0.27	12.7 ± 3.7	0.01 ± 0	0 ± 0	0 ± 0	13.07
FLIP07-87C	0.35 ± 0.01	11.59 ± 1.49	2.09 ± 0.31	0 ± 0	0 ± 0	14.02
ICC4936	0.35 ± 0.03	10.77 ± 1.87	1.42 ± 0.28	0 ± 0	0 ± 0	12.54
ILC2956	0.4 ± 0.04	15.82 ± 0.96	2.79 ± 0.2	0 ± 0	0.84 ± 0.44	19.86
ILC3856	0.5 ± 0.07	17.53 ± 0.97	2.62 ± 0.11	0 ± 0	0 ± 0	20.65
S95420	0.1 ± 0.02	10.11 ± 0.41	0.21 ± 0.02	0 ± 0	0 ± 0	10.42
DH27-4	0.52 ± 0.15	14.16 ± 1.56	0.5 ± 0.5	0 ± 0	0 ± 0	15.18
FLIP97-45C	0.33 ± 0.06	9.29 ± 0.75	0.63 ± 0.27	0 ± 0	0 ± 0	10.25
1707-9	0.71 ± 0.39	15.42 ± 6.12	3.06 ± 1.59	0 ± 0	0 ± 0	19.20
438-29	0.72 ± 0.15	16.73 ± 3.52	2.14 ± 0.42	0 ± 0	0.41 ± 0.41	20.00
1735-5	0.12 ± 0	8 ± 1.69	1.1 ± 0.21	0 ± 0	0 ± 0	9.22
CDC Leader	0.62 ± 0.06	13.25 ± 4.5	1.98 ± 0.74	0 ± 0	0 ± 0	15.84
X04TH41-4	0.26 ± 0.03	12.93 ± 2.36	1.7 ± 0.15	0 ± 0	0 ± 0	14.89
CDC Corinne	0.44 ± 0.02	6.75 ± 0.65	0.14 ± 0.07	0 ± 0	0 ± 0	7.34
2739-1	0.21 ± 0.07	12.46 ± 2.81	2.11 ± 0.52	0 ± 0	0 ± 0	14.78
1814-2	0.39 ± 0.05	10.31 ± 1.46	1.57 ± 0.22	0 ± 0	0 ± 0	12.26
AB06-156-2	0.09 ± 0.02	11.91 ± 0.77	0.32 ± 0.31	0 ± 0	0.27 ± 0.27	12.58
Myles	1.43 ± 0.05	24.93 ± 1.08	2.21 ± 0.32	0 ± 0	0.32 ± 0.32	28.89
AB06-159-2	0.03 ± 0.02	11.86 ± 0.83	0.01 ± 0	0 ± 0	0 ± 0	11.90
897-14	0.1 ± 0.06	5.93 ± 2.2	0.01 ± 0	0 ± 0	0 ± 0	6.04
Amit	0.11 ± 0.03	10.07 ± 2.43	1.24 ± 0.58	0 ± 0	0 ± 0	11.42
316B-42	0.46 ± 0.06	7.51 ± 1.49	1.17 ± 0.09	0 ± 0	0 ± 0	9.13
294T-16	0.25 ± 0.04	15.21 ± 5.84	2.66 ± 1.57	0.18 ± 0.18	1.09 ± 0.57	19.40
FLIP05-145C	0.53 ± 0.06	14.19 ± 1.6	2.54 ± 0.31	0 ± 0	0.42 ± 0.42	17.67
441-34	0.32 ± 0.17	13.14 ± 2.96	1.5 ± 0.24	0 ± 0	0.35 ± 0.35	15.32
93-120-63	0.34 ± 0.14	14.86 ± 2.34	1.87 ± 0.2	0 ± 0	0 ± 0	17.08
1701-1	0.19 ± 0.04	12.52 ± 1.95	1 ± 0.66	0 ± 0	0 ± 0	13.71
CDC Palmer (1041-3)	0.39 ± 0.11	4.64 ± 2.2	0.57 ± 0.29	0 ± 0	0 ± 0	5.60
1739-2	0.57 ± 0.34	9.51 ± 0.9	1.38 ± 0.19	0 ± 0	0 ± 0	11.47
ICC4475	0.62 ± 0.11	8.64 ± 0.98	0.77 ± 0.07	0 ± 0	0 ± 0	10.03
FLIP86-6C	0.29 ± 0.01	12.3 ± 3.34	0.01 ± 0	0 ± 0	0 ± 0	12.60
2085-1	0.32 ± 0.03	16.48 ± 1.19	3.3 ± 0.26	0 ± 0	0.48 ± 0.48	20.58
FLIP93-93	0.11 ± 0.01	3.43 ± 0.82	0.01 ± 0	0 ± 0	0 ± 0	3.55
2144-1	0.31 ± 0.1	10.14 ± 3.59	2.13 ± 0.87	0 ± 0	0.43 ± 0.43	13.01

FLIP97-677C	0.12 ± 0.03	3.93 ± 1.77	0.01 ± 0	0 ± 0	0 ± 0	4.06
2770-1	0.57 ± 0.22	4.5 ± 0.84	0.38 ± 0.03	0 ± 0	0 ± 0	5.46
ILC 3279	0.08 ± 0.02	8.35 ± 1.99	0.01 ± 0	0 ± 0	0 ± 0	8.44
X05TH47-3	0.08 ± 0.02	5.9 ± 2.45	0.01 ± 0	0 ± 0	0 ± 0	5.98
CDC Anna	0.37 ± 0.03	16.28 ± 2.95	1.94 ± 0.34	0 ± 0	0 ± 0	18.59
1569-1	0.09 ± 0.06	5.55 ± 4.76	0.01 ± 0	0 ± 0	0 ± 0	5.64
CDC Yuma	0.15 ± 0.01	5.9 ± 2.01	0.01 ± 0	0 ± 0	0 ± 0	6.06
846-1	0.09 ± 0.04	3.82 ± 1.43	0.01 ± 0	0 ± 0	0 ± 0	3.92
491-17	0.13 ± 0.07	15.84 ± 6.26	1.19 ± 1.18	0 ± 0	0.44 ± 0.44	17.60
356-6	0.52 ± 0.05	12.47 ± 0.92	2.19 ± 0.17	0 ± 0	0.38 ± 0.38	15.55
ICC14911	0.43 ± 0.08	19.84 ± 3.77	3.72 ± 0.75	0 ± 0	0 ± 0	23.99
FLIP05-43C	0.52 ± 0.04	13.38 ± 4.11	2.34 ± 0.62	0 ± 0	0 ± 0	16.24
ICC5124	0.33 ± 0.05	8.74 ± 1.24	1.54 ± 0.17	0 ± 0	0 ± 0	10.61
97-Indian2-107	0.31 ± 0.03	11.68 ± 1.71	1.35 ± 0.24	0 ± 0	0 ± 0	13.34
553-1	0.8 ± 0.08	14.01 ± 0.83	1.63 ± 0.14	0 ± 0	0 ± 0	16.44
1647-8	0.52 ± 0.12	18.17 ± 1.62	2.66 ± 0.34	0 ± 0	0 ± 0	21.35
1044-6	0.56 ± 0.08	6.9 ± 1.12	0.94 ± 0.39	0 ± 0	0 ± 0	8.41
FLIP05-162C	0.45 ± 0.05	16.23 ± 1.73	2.26 ± 0.13	0 ± 0	1.2 ± 0.6	20.14
FLIP81-293C	0.23 ± 0.02	5.07 ± 0.49	0.01 ± 0	0 ± 0	0 ± 0	5.31
2072-2	0.58 ± 0.15	16.78 ± 3.95	2.92 ± 0.73	0 ± 0	0 ± 0	20.28
2012 CP-BC-2-437	0.26 ± 0.04	12.76 ± 1.1	2.27 ± 0.32	0 ± 0	0 ± 0	15.29
1764-5	0.46 ± 0.28	10.61 ± 6.85	2.07 ± 1.52	0 ± 0	0.63 ± 0.63	13.77
Elixir	0.06 ± 0.03	5.84 ± 3.95	0.1 ± 0.09	0 ± 0	0 ± 0	6.00
1349-1	0.08 ± 0.02	11.81 ± 3.93	0.57 ± 0.57	0 ± 0	0.47 ± 0.47	12.93
CDC Luna	0.13 ± 0.02	17.21 ± 3.84	1.47 ± 0.77	0 ± 0	0 ± 0	18.81
x2001th 75-4	0.05 ± 0.02	10.27 ± 1.06	0.01 ± 0	0 ± 0	0 ± 0	10.32
CDC Jade	3.02 ± 0.23	35.91 ± 0.79	3.76 ± 0.62	0 ± 0	2.93 ± 0.28	45.63
x2001th 76-15	0.09 ± 0.02	9.75 ± 1.09	0.01 ± 0	0 ± 0	0 ± 0	9.85
FLIP98-135C	0.42 ± 0.15	12.37 ± 2.68	1.63 ± 0.34	0 ± 0	0 ± 0	14.43
CIABN-99PL27119	0.59 ± 0.05	10.85 ± 0.74	1.61 ± 0.08	0 ± 0	0 ± 0	13.05
FLIP05-80C	0.44 ± 0.16	19.04 ± 3.14	1.83 ± 0.95	0 ± 0	0.93 ± 0.47	22.23
CDC Cabri	0.2 ± 0.06	17.17 ± 2.68	0.47 ± 0.46	0 ± 0	0 ± 0	17.84
ILC202	0.37 ± 0.04	14.62 ± 2.94	2.65 ± 0.54	0 ± 0	0 ± 0	17.63
FLIP95-48C	0.12 ± 0.02	13.07 ± 2.66	1.25 ± 0.55	0 ± 0	0 ± 0	14.44
CA05-73-6	0.58 ± 0.08	7.13 ± 1.35	0.64 ± 0.41	0 ± 0	0 ± 0	8.35
95NN12	0.16 ± 0	4.86 ± 1.51	0.37 ± 0.37	0 ± 0	0 ± 0	5.40
561aS-18	0.37 ± 0.14	12.96 ± 1.91	1.6 ± 0.42	0 ± 0	0 ± 0	14.93
1045-1	0.52 ± 0.06	6.2 ± 1.24	0.01 ± 0	0 ± 0	0 ± 0	6.73
1778-2	0.13 ± 0.11	8.71 ± 1.88	0.83 ± 0.63	0 ± 0	0 ± 0	9.67
FLIP82-150C	0.33 ± 0.09	5.21 ± 1.28	0.01 ± 0	0 ± 0	0 ± 0	5.55
FLIP07-40C	0.24 ± 0.08	12.76 ± 4.25	1.93 ± 0.8	0 ± 0	0 ± 0	14.93
2382-3	0.7 ± 0.2	22.4 ± 4.33	3.85 ± 1.14	0 ± 0	0 ± 0	26.94
FLIP93-58C	0.12 ± 0.04	7.74 ± 5.05	0.79 ± 0.79	0 ± 0	0 ± 0	8.66
2012 CP-BC-2-472	0.39 ± 0.13	13.7 ± 3.68	2.46 ± 0.74	0 ± 0	0 ± 0	16.55
2223-2	0.29 ± 0.01	15.13 ± 0.49	3.24 ± 0.33	0 ± 0	0.46 ± 0.46	19.12

ILC482	0.16 ± 0.16	10.39 ± 5.63	0.9 ± 0.89	0 ± 0	0 ± 0	11.45
2293-3	0.18 ± 0.11	7.49 ± 2.28	1.09 ± 0.59	0 ± 0	0.4 ± 0.4	9.16
X05TH20-2	0 ± 0	2.44 ± 1.02	0.01 ± 0	0 ± 0	0 ± 0	2.45
95168-64	0.11 ± 0.03	11.91 ± 0.83	0.61 ± 0.6	0 ± 0	0 ± 0	12.62
1136-3	0.06 ± 0.01	5.92 ± 0.49	0.01 ± 0	0 ± 0	0 ± 0	5.99
242-2	0.14 ± 0.01	14.52 ± 0.95	1.53 ± 0.19	0 ± 0	0 ± 0	16.18
548aS-20	0.17 ± 0.03	3.64 ± 0.18	0.01 ± 0	0 ± 0	0 ± 0	3.81
FLIP95-56C	0.57 ± 0.1	12.4 ± 1.69	1.73 ± 0.23	0 ± 0	0 ± 0	14.70
CDC Orion	0.56 ± 0.07	13.67 ± 1.55	1.96 ± 0.23	0 ± 0	0.42 ± 0.42	16.60
BS1-43	0.24 ± 0.08	19.09 ± 1.63	0.46 ± 0.45	0 ± 0	0.96 ± 0.52	20.74
FLIP07-1C	0.5 ± 0.07	15.79 ± 2.32	3.43 ± 0.52	0 ± 0	0 ± 0	19.72
FLIP07-25C	0.53 ± 0.14	16.83 ± 3.51	3.22 ± 0.61	0 ± 0	0.38 ± 0.38	20.96
CDC Consul (603-3)	0.55 ± 0.09	6.67 ± 1.85	0.01 ± 0	0 ± 0	0 ± 0	7.23
GPE094	0.69 ± 0.06	14.63 ± 1.19	1.86 ± 0.2	0 ± 0	0 ± 0	17.18
1683-3	0.27 ± 0.08	12.48 ± 2.21	2.22 ± 0.5	0 ± 0	0 ± 0	14.96
1632-7	0.27 ± 0.08	13.9 ± 2.44	2.06 ± 0.38	0 ± 0	0 ± 0	16.24
FLIP87-45C	0.31 ± 0.05	12.39 ± 4.55	0.01 ± 0	0 ± 0	0 ± 0	12.71
2385-1	0.31 ± 0.17	10.24 ± 2.86	1.7 ± 0.6	0 ± 0	0 ± 0	12.25
FLIP97-137C	0.09 ± 0.02	15.88 ± 4.13	2.01 ± 0.41	0 ± 0	0.38 ± 0.38	18.35
2012 CP-BC-2-434	0.23 ± 0.12	7.97 ± 4.3	1.44 ± 0.78	0 ± 0	0 ± 0	9.64
1757-8	0.29 ± 0.05	9.9 ± 0.97	1.76 ± 0.12	0 ± 0	0 ± 0	11.96
713-13	0.11 ± 0.11	10.28 ± 2.39	1.13 ± 0.48	0 ± 0	0 ± 0	11.53
2239-4	0.11 ± 0.06	11.64 ± 5.11	1.33 ± 0.41	0 ± 0	0.7 ± 0.35	13.78
CDC Chico	0.12 ± 0.01	8.92 ± 0.96	0.01 ± 0	0 ± 0	0 ± 0	9.05
AB06-106-2	0.02 ± 0.02	6.17 ± 3.01	0.54 ± 0.53	0 ± 0	0 ± 0	6.73
95177-47	0.11 ± 0.02	9.69 ± 2.85	1.05 ± 0.57	0 ± 0	0 ± 0	10.85
1220-1	0.23 ± 0.05	10.06 ± 5.28	0.01 ± 0	0 ± 0	0 ± 0	10.30
97-Indian2-112	0.19 ± 0	13.4 ± 2.1	2.03 ± 0.21	0 ± 0	0 ± 0	15.63
x2001th 169-3	0.11 ± 0.04	3.6 ± 0.37	0.01 ± 0	0 ± 0	0 ± 0	3.72
381T-4	0.38 ± 0.02	5.72 ± 0.78	0.01 ± 0	0 ± 0	0 ± 0	6.10
413-2	0.27 ± 0.07	15.78 ± 3.02	0.01 ± 0	0 ± 0	0.3 ± 0.3	16.35
492-24	0.57 ± 0.05	11.51 ± 0.81	1.59 ± 0.12	0 ± 0	0 ± 0	13.67
612-4	0.51 ± 0.01	4.91 ± 0.67	0.28 ± 0.28	0 ± 0	0 ± 0	5.70
1702-1	0.27 ± 0.03	13.82 ± 2.3	2.35 ± 0.38	0 ± 0	0 ± 0	16.44
FLIP84-92C	0.38 ± 0.03	4.57 ± 1.66	0.01 ± 0	0 ± 0	0 ± 0	4.96
1414-2	0.13 ± 0.03	8.7 ± 1.53	1.43 ± 0.31	0 ± 0	0 ± 0	10.26
2393-2	0.28 ± 0.05	14.92 ± 0.61	2.62 ± 0.31	0 ± 0	0 ± 0	17.83
FLIP 09-6C	0.34 ± 0.06	15.26 ± 2.07	2.79 ± 0.57	0 ± 0	0 ± 0	18.39
ILC 72	0.08 ± 0.04	17 ± 2.39	0.45 ± 0.44	0 ± 0	0 ± 0	17.53
1957-1	0.2 ± 0.01	6.32 ± 0.78	1.31 ± 0.14	0 ± 0	0 ± 0	7.83
1173-1	0.06 ± 0.03	3.94 ± 0.99	0.01 ± 0	0 ± 0	0 ± 0	4.01
FLIP05-46C	0.38 ± 0.1	14.64 ± 3.91	2.6 ± 0.71	0 ± 0	0 ± 0	17.61
CA05-75-45	0.19 ± 0.12	9.33 ± 4.44	0.01 ± 0	0 ± 0	0 ± 0	9.52
FLIP06-116C	0.36 ± 0.17	13.3 ± 4.55	2.23 ± 0.72	0 ± 0	0 ± 0	15.89
1460-2	0 ± 0	5.03 ± 1.24	0.01 ± 0	0 ± 0	0 ± 0	5.03

FLIP07-39C	0.39 ± 0.04	10.43 ± 0.61	1.7 ± 0.07	0 ± 0	0 ± 0	12.53
x2001th 77-5	0.15 ± 0.03	4.39 ± 1.59	0.01 ± 0	0 ± 0	0 ± 0	4.54
ICC1069	0.73 ± 0.06	21.63 ± 1.21	2.63 ± 0.38	0 ± 0	1.55 ± 0.33	26.53
FLIP98-136C	0.2 ± 0.08	20.42 ± 1.15	0.01 ± 0	0 ± 0	0.34 ± 0.34	20.96

Table A4. The concentration (μg g^{-1}) of different seed carotenoids including violaxanthin, lutein, zeaxanthin, β-carotene, β-cryptoxanthin, and total carotenoids ± Se measured by HPLC in 120 $F_{2:3}$ chickpea seeds in cross between CDC Jade and CDC Frontier grown in greenhouse. Three different cotyledon classes including Y (yellow), G (green) and S (segregating) has shown for each family.

Family	Cotyledon	Violaxanthin	Lutein	Zeaxanthin	β-Cryptoxanthin	β-Carotene	Total
1	Y	0.72 ± 0.04	21.39 ± 0.91	5.72 ± 0.37	0 ± 0	1.04 ± 0.3	28.88
2	S	1.48 ± 0.57	20.83 ± 0.96	3.55 ± 0.22	0.36 ± 0.31	0.76 ± 0.66	27
3	G	3.42 ± 0.05	27.44 ± 0.1	4.45 ± 0.04	0.92 ± 0.08	3.19 ± 0.04	39.42
4	Y	0.57 ± 0.16	15.68 ± 4.71	4.38 ± 1.31	0 ± 0	0.3 ± 0.26	20.93
5	Y	1.18 ± 0.07	26.21 ± 0.37	6.08 ± 0.27	0 ± 0	0 ± 0	33.47
6	S	2.64 ± 1.03	30.62 ± 1.48	6.07 ± 0.72	0.39 ± 0.34	2.03 ± 0.83	41.76
7	S	2.44 ± 0.85	27.62 ± 0.49	5.06 ± 0.74	0.45 ± 0.39	1.52 ± 0.71	37.08
8	Y	1.04 ± 0.09	24.07 ± 1.51	4.88 ± 0.25	0 ± 0	0.36 ± 0.18	30.34
9	Y	0.85 ± 0.01	20.97 ± 0.06	2.07 ± 0.02	0 ± 0	0.88 ± 0.01	24.77
10	G	1.5 ± 0.03	23.74 ± 0.02	3.3 ± 0.05	0 ± 0	2.39 ± 0.01	30.94
11	S	2.59 ± 0.95	34.26 ± 0.77	5.86 ± 0.49	0.57 ± 0.49	1.9 ± 0.82	45.17
12	Y	1.08 ± 0.03	20.55 ± 0.9	4.29 ± 0.26	0 ± 0	0 ± 0	25.92
13	Y	0.08 ± 0.01	15.15 ± 0.19	1.19 ± 0.05	0 ± 0	0 ± 0	16.42
14	S	0.58 ± 0.01	18.71 ± 0.13	1.68 ± 0.02	0 ± 0	0 ± 0	20.97
15	Y	1 ± 0.03	18.65 ± 0.85	3.39 ± 0.1	0 ± 0	0.29 ± 0.26	23.33
16	Y	0.75 ± 0.02	20.56 ± 0.13	1.96 ± 0.01	0 ± 0	0.51 ± 0.15	23.78
17	S	1.51 ± 0.49	19.3 ± 0.55	3.19 ± 0.65	0.3 ± 0.26	0.61 ± 0.53	24.9
18	S	1.96 ± 0.67	24.33 ± 1.6	3.57 ± 0.21	0.51 ± 0.44	0.95 ± 0.82	31.32
19	S	1.89 ± 0.75	23.56 ± 1.41	4.64 ± 0.37	0.44 ± 0.38	1.07 ± 0.71	31.6
20	S	1.26 ± 0.04	24.95 ± 0.43	4.1 ± 0.26	0 ± 0	0 ± 0	30.31
21	S	2.18 ± 0.65	25.13 ± 1.19	4.38 ± 0.44	0.45 ± 0.39	1.81 ± 0.49	33.96
22	S	1.72 ± 0.51	20.7 ± 1.13	3.83 ± 0.17	0.35 ± 0.3	0.77 ± 0.67	27.37
23	G	4.71 ± 0.42	35.37 ± 2.58	5.62 ± 0.17	1.99 ± 0.42	3.85 ± 0.01	51.54
24	S	1.21 ± 0.33	15.3 ± 0.67	3.46 ± 0.61	0 ± 0	0.4 ± 0.34	20.37
25	G	3.2 ± 0.01	26.66 ± 0.19	4.17 ± 0.06	0.67 ± 0.03	3 ± 0.02	37.69
26	Y	0.97 ± 0.11	24.41 ± 0.64	3.77 ± 0.2	0 ± 0	0 ± 0	29.15
27	Y	0.28 ± 0.07	15.99 ± 0.06	1.41 ± 0.01	0 ± 0	0 ± 0	17.68
28	Y	0.98 ± 0.01	21.01 ± 0.75	4.6 ± 0.06	0 ± 0	0 ± 0	26.59
29	Y	0.52 ± 0.01	17.72 ± 0.04	1.6 ± 0.01	0 ± 0	0 ± 0	19.85
30	Y	1.07 ± 0.06	22.14 ± 0.75	5.96 ± 0.35	0 ± 0	0 ± 0	29.18
31	S	1.88 ± 0.59	21.98 ± 0.57	3.87 ± 0.14	0.47 ± 0.41	0.87 ± 0.76	29.08
32	S	2.08 ± 0.88	23.16 ± 1.24	3.82 ± 0.45	0.4 ± 0.35	0.87 ± 0.75	30.33
33	S	1.37 ± 0.29	17.37 ± 0.42	4.22 ± 0.49	0.24 ± 0.21	0.46 ± 0.39	23.67
34	S	1.73 ± 0.31	22.75 ± 1.17	3.64 ± 0.18	0.3 ± 0.26	0.86 ± 0.46	29.29
35	G	1.31 ± 0.04	23.51 ± 0.07	3.03 ± 0.02	0 ± 0	2.3 ± 0.02	30.16
36	Y	1.14 ± 0.24	26.27 ± 0.86	6.5 ± 0.14	0 ± 0	0.22 ± 0.19	34.13
37	S	4.22 ± 1.76	30.84 ± 3.27	3.82 ± 0.33	1.2 ± 0.73	2.1 ± 1.23	42.17
38	S	2.62 ± 0.78	31.22 ± 2.99	4.92 ± 0.19	0.59 ± 0.51	1.66 ± 0.76	41.02
39	Y	1.27 ± 0.06	24.19 ± 1.2	7.77 ± 0.16	0 ± 0	0 ± 0	33.23

40	Y	3.07 ± 0.59	36.12 ± 1.67	6.42 ± 0.17	0 ± 0	0 ± 0	45.62
41	S	2.19 ± 0.48	24.18 ± 1.29	5 ± 0.43	0.53 ± 0.45	0.7 ± 0.6	32.6
42	S	2.67 ± 0.62	27.66 ± 2.06	5 ± 0.38	0.48 ± 0.41	0.81 ± 0.4	36.62
43	S	0.46 ± 0.01	16.52 ± 0.04	1.49 ± 0.02	0 ± 0	0 ± 0	18.46
44	Y	0.5 ± 0	17.17 ± 0.05	1.56 ± 0	0 ± 0	0 ± 0	19.23
45	G	4.17 ± 0.27	26.87 ± 1.2	4.1 ± 0.16	2.73 ± 0.18	1.79 ± 0.07	39.67
46	Y	0 ± 0	14.28 ± 0.11	0.66 ± 0.12	0 ± 0	0 ± 0	14.94
47	S	1.7 ± 0.69	23.65 ± 1.33	3.25 ± 0.44	0 ± 0	0.85 ± 0.74	29.46
48	G	3.39 ± 0.25	21.39 ± 1.11	3.26 ± 0.22	0 ± 0	1.91 ± 0.23	29.95
49	G	3.51 ± 0.22	23.71 ± 0.98	3.86 ± 0.17	0 ± 0	2.38 ± 0.2	33.45
50	Y	1.47 ± 0.06	26.47 ± 1.9	6.94 ± 0.91	0 ± 0	0.94 ± 0.28	35.81
51	S	1.87 ± 0.4	23.23 ± 1.18	3.65 ± 0.2	0 ± 0	0.57 ± 0.49	29.31
52	S	3.09 ± 0.89	37.86 ± 2.08	6.82 ± 0.17	0 ± 0	1.85 ± 0.8	49.62
53	S	2.41 ± 0.63	25.09 ± 1.31	4.48 ± 0.19	0.55 ± 0.48	1.11 ± 0.68	33.65
54	S	2.63 ± 0.98	27.92 ± 2.33	5.99 ± 0.25	0 ± 0	1.52 ± 0.99	38.06
55	S	1.85 ± 1.17	26.18 ± 2.95	5.22 ± 0.8	1.02 ± 0.89	1.49 ± 1.29	35.76
56	Y	1.84 ± 0.12	28.38 ± 1.55	4.42 ± 0.35	0 ± 0	0.34 ± 0.29	34.98
57	Y	2.86 ± 0.17	36.91 ± 1.41	6.08 ± 0.49	0 ± 0	0 ± 0	45.85
58	S	3.06 ± 0.57	23.82 ± 0.68	4.46 ± 0.24	0 ± 0	1.09 ± 0.61	32.42
59	G	4.23 ± 0.24	25.43 ± 1.59	5.28 ± 0.59	1.06 ± 0.24	1.83 ± 0.24	37.85
60	S	0.98 ± 0.12	21 ± 0.39	3.29 ± 0.15	0 ± 0	0 ± 0	25.28
61	Y	1.2 ± 0.07	24.46 ± 1.28	5.38 ± 0.58	0 ± 0	0 ± 0	31.03
62	S	3.82 ± 0.71	32.97 ± 1.72	5.08 ± 0.39	0.8 ± 0.7	1.14 ± 0.99	43.81
63	S	0.88 ± 0	21.4 ± 0.07	2.19 ± 0.03	0 ± 0	0.94 ± 0.01	25.4
64	S	2.37 ± 0.68	25.84 ± 0.93	5.39 ± 0.53	0 ± 0	0.75 ± 0.65	34.35
65	G	4.88 ± 0.31	31.19 ± 1.32	4.49 ± 0.21	0 ± 0	3.27 ± 0.2	43.83
66	S	2.52 ± 1.08	33.73 ± 4.8	5.08 ± 0.56	0 ± 0	1.23 ± 1.07	42.57
67	S	1.77 ± 0.64	22.22 ± 1.45	5.22 ± 0.54	0 ± 0	0.63 ± 0.54	29.83
68	S	2.34 ± 0.89	29.11 ± 1.18	4.91 ± 0.5	0 ± 0	0.94 ± 0.81	37.3
69	S	2.19 ± 0.85	30.49 ± 0.65	6.87 ± 0.96	0 ± 0	0.97 ± 0.84	40.53
70	S	1.73 ± 0.63	20.85 ± 2.37	4.09 ± 0.3	0 ± 0	0.82 ± 0.71	27.49
71	Y	1.93 ± 0.14	30.11 ± 0.18	7.63 ± 0.44	0 ± 0	0 ± 0	39.67
72	S	2.6 ± 1.05	23.53 ± 0.95	2.63 ± 0.77	0 ± 0	0.85 ± 0.73	29.61
73	S	2.18 ± 0.57	26.87 ± 1.08	4.97 ± 0.23	0 ± 0	0.73 ± 0.63	34.75
74	Y	3.51 ± 0.46	34.04 ± 0.82	5.15 ± 0.94	0 ± 0	0 ± 0	42.7
75	S	1.82 ± 0.58	24.54 ± 1.45	5.55 ± 0.63	0 ± 0	0.58 ± 0.51	32.5
76	Y	1.42 ± 0.15	23.75 ± 1.76	3.28 ± 0.5	0 ± 0	0 ± 0	28.45
77	S	3.03 ± 0.54	34.67 ± 0.65	4.32 ± 0.32	0 ± 0	0.75 ± 0.65	42.77
78	S	2.6 ± 0.71	28.39 ± 2.08	2.39 ± 0.83	0 ± 0	0.78 ± 0.68	34.16
79	G	3.04 ± 0.03	25.54 ± 0.06	3.9 ± 0.04	0.45 ± 0.01	2.7 ± 0.01	35.63
80	G	1.07 ± 0.02	22.55 ± 0.14	2.87 ± 0.01	0 ± 0	2.2 ± 0.02	28.68
81	S	2.61 ± 0.41	30.45 ± 2.13	5.7 ± 0.7	0 ± 0	1.28 ± 0.38	40.05
82	G	5.23 ± 0.87	29.43 ± 2.09	1.14 ± 0.5	0.14 ± 0.12	1.66 ± 0.53	37.6
83	S	2.02 ± 0.97	24.38 ± 2.52	6.16 ± 1.07	0 ± 0	0.99 ± 0.86	33.54

84	Y	1.19 ± 0.05	24.22 ± 0.51	7.27 ± 0.16	0 ± 0	0 ± 0	32.67
85	G	2.15 ± 0.17	24.27 ± 0.04	3.56 ± 0.03	0.13 ± 0.07	2.51 ± 0.04	32.62
86	S	2.38 ± 0.71	33.78 ± 1.64	5.68 ± 1.05	0 ± 0	1.82 ± 1.23	43.66
87	G	3.65 ± 0.03	28.55 ± 0.07	4.74 ± 0.12	1.18 ± 0.01	3.45 ± 0.11	41.57
88	Y	1.15 ± 0.26	21.91 ± 1.36	6.09 ± 0.44	0 ± 0	0 ± 0	29.16
89	S	1.98 ± 0.75	25.44 ± 1.82	5.53 ± 0.99	0 ± 0	0.76 ± 0.66	33.7
90	S	2.35 ± 0.73	23.46 ± 0.8	5.24 ± 0.55	0 ± 0	0.81 ± 0.47	31.86
91	G	2.81 ± 0.05	24.6 ± 0.1	3.74 ± 0.02	0.37 ± 0.02	2.67 ± 0.01	34.18
92	Y	1.59 ± 0.2	32.18 ± 0.66	6.8 ± 0.47	0 ± 0	0 ± 0	40.57
93	G	4.38 ± 0.14	28.88 ± 0.95	3.48 ± 0.36	0 ± 0	3.08 ± 0.15	39.82
94	S	1.84 ± 0.5	23.28 ± 1.23	4.14 ± 0.53	0 ± 0	0.68 ± 0.59	29.94
95	S	1.86 ± 0.69	27.33 ± 2.04	4.2 ± 0.26	0 ± 0	0.67 ± 0.58	34.06
96	Y	0.93 ± 0.01	21.7 ± 0.09	2.46 ± 0.03	0 ± 0	1.23 ± 0.13	26.31
97	Y	1.08 ± 0.27	24.17 ± 0.31	6.55 ± 0.15	0.28 ± 0.24	0.4 ± 0.35	32.48
98	S	2.38 ± 1.04	34.75 ± 1.72	6.2 ± 0.98	0 ± 0	1.38 ± 0.93	44.7
99	Y	2.34 ± 0.19	31.48 ± 0.32	5.88 ± 0.26	0 ± 0	0.47 ± 0.24	40.17
100	Y	1.33 ± 0.1	21.33 ± 1.39	3.65 ± 0.28	0 ± 0	0 ± 0	26.3
101	S	3.21 ± 1.13	35.5 ± 0.76	5.2 ± 0.8	0.45 ± 0.39	2.05 ± 0.7	46.41
102	S	1.78 ± 0.55	18.9 ± 0.32	5.14 ± 0.64	0 ± 0	0.5 ± 0.43	26.31
103	Y	0.69 ± 0	19.41 ± 0.08	1.85 ± 0.03	0 ± 0	0 ± 0	21.94
104	Y	1.74 ± 0.28	28.37 ± 1.11	3.89 ± 0.31	0 ± 0	0 ± 0	34
105	S	3.45 ± 1.11	45.47 ± 1.78	6.33 ± 0.71	0.61 ± 0.53	2.28 ± 0.82	58.14
106	G	6.4 ± 0.42	35.56 ± 2.27	6.13 ± 0.66	0.9 ± 0.17	3.04 ± 0.31	52.04
107	S	2.07 ± 0.37	18.13 ± 0.69	3.1 ± 0.16	0 ± 0	0.45 ± 0.39	23.75
108	Y	2.16 ± 0.13	33.27 ± 1.87	4.64 ± 0.72	0 ± 0	1.03 ± 0.14	41.09
109	S	3.06 ± 0.75	28.42 ± 0.56	3.81 ± 0.26	0 ± 0	1.39 ± 0.5	36.67
110	G	1 ± 0.01	22.17 ± 0.04	2.69 ± 0.03	0 ± 0	1.84 ± 0.1	27.69
111	S	3.6 ± 0.84	43.74 ± 1.56	5 ± 0.64	0.47 ± 0.41	2.26 ± 0.65	55.07
112	Y	2.39 ± 0.12	32.02 ± 1.35	3.77 ± 0.19	0 ± 0	0.23 ± 0.2	38.41
113	S	5.5 ± 1.11	43.84 ± 1.48	4.52 ± 0.22	0 ± 0	2.52 ± 0.7	56.38
114	S	2.86 ± 0.8	30.57 ± 1.65	3.12 ± 0.2	0 ± 0	1.12 ± 0.54	37.67
115	Y	1.76 ± 0.2	27.28 ± 1.03	4.12 ± 0.28	0 ± 0	1.01 ± 0.09	34.18
116	G	3.14 ± 0.01	25.92 ± 0.05	4.01 ± 0.01	0.55 ± 0.02	2.86 ± 0.03	36.47
117	S	2.69 ± 0.61	24.99 ± 0.91	2.46 ± 0.22	0.41 ± 0.35	1.46 ± 0.54	32.01
118	Y	1.57 ± 0.14	22.11 ± 1.06	3.71 ± 0.4	0 ± 0	0.56 ± 0.29	27.96
119	S	3.98 ± 1.25	39.35 ± 2.12	4.47 ± 0.33	0.5 ± 0.43	2.4 ± 0.75	50.69
120	Y	2.02 ± 0.11	24.95 ± 0.63	3.45 ± 0.16	0 ± 0	0.84 ± 0.08	31.26

Table A5. The concentration (μg g^{-1}) of different seed carotenoids including violaxanthin, lutein, zeaxanthin, β-carotene, β-cryptoxanthin, and total carotenoids ± Se measured by HPLC in 120 $F_{2:3}$ chickpea seeds in cross between CDC Cory and CDC Jade grown in greenhouse. Three different cotyledon classes including Y (yellow), G (green) and S (segregating) has shown for each family.

Family	Cotyledon	Violaxanthin	Lutein	Zeaxanthin	β-Cryptoxanthin	β-Carotene	Total
1	S	3.13 ± 0.39	50.96 ± 0.57	4.67 ± 0.49	0.81 ± 0.7	2.12 ± 1.39	61.69
2	G	3.2 ± 0.14	45.41 ± 1.69	3.39 ± 0.14	0.44 ± 0.38	4 ± 0.33	56.44
3	Y	0.65 ± 0.01	12.36 ± 2.52	2.46 ± 0.02	0 ± 0	0 ± 0	15.47
4	Y	1.05 ± 0.01	26.13 ± 0.13	3.46 ± 0.02	0.59 ± 0.02	1.81 ± 0.01	33.03
5	S	1.93 ± 0.16	43.01 ± 3.69	6.12 ± 0.74	0.43 ± 0.37	1.72 ± 1.03	53.21
6	Y	1.88 ± 0.09	43.01 ± 1.97	7.85 ± 0.39	0 ± 0	0.4 ± 0.35	53.14
7	S	3.17 ± 0.28	44.2 ± 0.46	5.05 ± 0.48	1.24 ± 0.22	2.62 ± 0.23	56.28
8	S	4.01 ± 0.24	63.23 ± 1.21	6.81 ± 0.65	1.27 ± 0.57	2.31 ± 0.9	77.63
9	S	1.74 ± 0.09	40.18 ± 4.04	5.35 ± 1.15	0.57 ± 0.49	1.75 ± 0.64	49.59
10	S	2.99 ± 0.39	48.56 ± 3.49	5.06 ± 0.66	0.9 ± 0.78	2.45 ± 1.11	59.97
11	S	3.13 ± 0.47	42.21 ± 1.16	4.2 ± 1.4	0 ± 0	0.35 ± 0.31	49.9
12	S	2.77 ± 0.12	45.85 ± 2.15	2.28 ± 0.74	0 ± 0	1.61 ± 0.2	52.51
13	S	3.64 ± 0.65	58.43 ± 2.88	3.73 ± 1.15	0.4 ± 0.34	1.6 ± 1	67.79
14	S	2.75 ± 0.35	51.17 ± 4.28	4.01 ± 0.87	0.56 ± 0.49	1.7 ± 1.26	60.19
15	S	1.57 ± 0.2	45.51 ± 3.01	3.83 ± 1.12	0.45 ± 0.39	1.88 ± 0.95	53.25
16	S	2.51 ± 0.13	43.55 ± 1.31	4.19 ± 0.27	0.42 ± 0.37	1.48 ± 0.7	52.15
17	G	3.17 ± 0.16	41.73 ± 1	5.52 ± 0.15	2.18 ± 0.21	4.02 ± 0.23	56.62
18	S	3.83 ± 0.4	62.6 ± 3.77	4.22 ± 0.24	0.82 ± 0.54	2.26 ± 0.68	73.75
19	G	2.42 ± 0.1	37.46 ± 2.86	3.77 ± 0.34	0 ± 0	2.4 ± 0.42	46.06
20	G	1.44 ± 0.01	32.59 ± 0.23	4.84 ± 0.02	1.41 ± 0	3.8 ± 0.05	44.08
21	Y	1.61 ± 0.19	44.44 ± 1.14	6.26 ± 0.13	0 ± 0	0.14 ± 0.12	52.45
22	S	2.69 ± 0.49	50.78 ± 4.66	4.74 ± 0.57	0.47 ± 0.41	0.92 ± 0.63	59.61
23	S	2.07 ± 0.22	44.99 ± 2.66	4.28 ± 0.48	0 ± 0	2.48 ± 0.32	53.82
24	Y	0.98 ± 0.01	23.83 ± 0.08	3.21 ± 0.01	0 ± 0	1.32 ± 0.06	29.34
25	Y	2.22 ± 0.28	40.74 ± 2.76	7.41 ± 0.49	0 ± 0	0.59 ± 0.19	50.97
26	S	3.74 ± 0.39	47.6 ± 2.16	4.8 ± 0.62	0.66 ± 0.37	1.88 ± 0.52	58.69
27	Y	1.09 ± 0.01	26.62 ± 0.13	3.63 ± 0.02	0.76 ± 0.03	2.11 ± 0.07	34.21
28	Y	0.93 ± 0	23.56 ± 0.05	3.04 ± 0.04	0 ± 0	0.99 ± 0.03	28.52
29	S	2.5 ± 0.22	42.34 ± 1.65	5.93 ± 0.47	0.82 ± 0.71	1.68 ± 0.71	53.26
30	Y	0.73 ± 0.01	19.15 ± 0.17	2.68 ± 0.05	0 ± 0	0 ± 0	22.56
31	G	2.13 ± 0.18	39.3 ± 0.36	8.48 ± 0.55	3.93 ± 0.15	6.23 ± 0.15	60.07
32	G	1.3 ± 0.01	29.22 ± 0.18	4.11 ± 0.01	1.19 ± 0.02	3.17 ± 0.03	39
33	S	2.17 ± 0.28	46.42 ± 0.82	5.14 ± 0.64	0.62 ± 0.33	1.94 ± 0.48	56.29
34	Y	1.79 ± 0.1	47.94 ± 1.79	5.68 ± 0.16	0.28 ± 0.24	2.29 ± 0.58	57.97
35	S	1.83 ± 0.11	41.89 ± 2.08	5.52 ± 0.32	0 ± 0	2.53 ± 0.93	51.78
36	G	1.56 ± 0.01	34.28 ± 0.07	5.62 ± 0.06	1.82 ± 0.05	4.18 ± 0.04	47.47
37	S	2.93 ± 0.26	47.67 ± 1.89	5.08 ± 0.64	0.27 ± 0.24	1.92 ± 0.61	57.86
38	Y	1.67 ± 0.14	45.61 ± 1.96	6.9 ± 0.14	0 ± 0	0.76 ± 0.23	54.94
39	S	2.01 ± 0.37	35.68 ± 0.82	4.16 ± 0.83	0.38 ± 0.33	1.31 ± 0.67	43.55

40	G	2.98 ± 0.09	39.67 ± 0.96	2.94 ± 0.14	2.51 ± 0.05	4.43 ± 0.37	52.53
41	Y	0.43 ± 0.03	1.26 ± 0.44	0.27 ± 0.02	0 ± 0	0 ± 0	1.96
42	S	2.89 ± 0.42	43.98 ± 2.5	4.54 ± 0.35	0.66 ± 0.58	2.26 ± 0.63	54.34
43	G	1.62 ± 0.02	34.85 ± 0.21	6.08 ± 0.09	2.05 ± 0.02	4.63 ± 0.08	49.23
44	Y	1.87 ± 0.11	46.37 ± 3.56	6.62 ± 0.42	0.21 ± 0.18	1.1 ± 0.23	56.17
45	Y	1.56 ± 0.02	38.53 ± 1.88	7.26 ± 0.44	0 ± 0	0.74 ± 0.24	48.08
46	S	3.33 ± 0.49	55.36 ± 3.5	4.79 ± 0.58	0.46 ± 0.23	2.41 ± 0.66	66.35
47	S	2.36 ± 0.37	46.12 ± 3.9	5.83 ± 0.48	0.72 ± 0.4	2.78 ± 1.17	57.8
48	Y	1.67 ± 0.34	38.32 ± 0.8	7.27 ± 0.34	0 ± 0	1.11 ± 0.13	48.38
49	S	2.72 ± 0.27	43.99 ± 1.3	5.16 ± 0.5	0.67 ± 0.41	1.83 ± 0.83	54.37
50	Y	1.76 ± 0.25	43.96 ± 1.07	7.01 ± 0.44	0 ± 0	0.69 ± 0.21	53.42
51	S	2.11 ± 0.23	43.09 ± 0.77	5.93 ± 0.67	0.78 ± 0.49	2.54 ± 0.82	54.45
52	S	2.87 ± 0.68	47.87 ± 2.98	5.68 ± 0.62	1.21 ± 0.79	3.17 ± 1.58	60.8
53	G	1.34 ± 0	30.21 ± 0.14	4.38 ± 0.06	1.27 ± 0.01	3.39 ± 0.02	40.58
54	S	2.38 ± 0.26	48.43 ± 1.54	5.48 ± 0.78	0.3 ± 0.26	1.84 ± 1.09	58.43
55	S	2.22 ± 0.22	41.4 ± 2	6.05 ± 0.67	0.67 ± 0.58	1.29 ± 1.11	51.63
56	Y	1.62 ± 0.16	42.31 ± 2.01	8.35 ± 0.29	0 ± 0	0.54 ± 0.27	52.82
57	Y	1.44 ± 0.42	44.21 ± 1.15	7.34 ± 0.51	0 ± 0	0 ± 0	52.99
58	S	2 ± 0.17	44.84 ± 1.85	6.67 ± 1.22	0.35 ± 0.3	1.35 ± 0.64	55.2
59	S	1.9 ± 0.35	40.52 ± 0.5	5.89 ± 0.71	0.45 ± 0.39	1.16 ± 0.76	49.92
60	S	2.55 ± 0.35	59.92 ± 1.27	7.75 ± 0.32	0.53 ± 0.37	2.32 ± 0.55	73.06
61	S	2.1 ± 0.7	38.02 ± 1.09	4.73 ± 0.65	0.77 ± 0.67	1.49 ± 1.29	47.11
62	S	1.53 ± 0.3	34.33 ± 7.95	4.59 ± 1.2	0 ± 0	0.47 ± 0.4	40.91
63	S	2.6 ± 0.13	46.73 ± 1.2	4.84 ± 0.27	0 ± 0	2.7 ± 0.97	56.88
64	S	2.46 ± 0.25	41.63 ± 0.98	4.99 ± 0.23	0 ± 0	1.71 ± 0.62	50.78
65	S	2.9 ± 0.11	53.57 ± 1.46	4.98 ± 0.77	0.36 ± 0.31	2.32 ± 0.88	64.13
66	S	2.29 ± 0.45	45.84 ± 4.18	4.38 ± 0.29	0.89 ± 0.77	2.31 ± 2	55.71
67	S	1.93 ± 0.26	39.04 ± 1.24	3.79 ± 1.11	0 ± 0	0.83 ± 0.72	45.6
68	S	1.59 ± 0.17	33.73 ± 3.01	3.33 ± 1.15	0.45 ± 0.23	1.96 ± 0.15	41.08
69	Y	0.6 ± 0.01	6.27 ± 0.32	2.06 ± 0.13	0 ± 0	0 ± 0	8.93
70	Y	2.76 ± 0.45	39.89 ± 2.1	4.64 ± 0.27	0 ± 0	0 ± 0	47.29
71	S	2.64 ± 0.11	51.34 ± 2.5	6.26 ± 0.34	0 ± 0	1.4 ± 1.22	61.64
72	G	2.8 ± 0.27	50.11 ± 2.17	5.41 ± 0.52	1.15 ± 0.35	4.89 ± 0.5	64.35
73	G	1.14 ± 0.01	27.28 ± 0.02	3.73 ± 0.01	0.86 ± 0.01	2.4 ± 0.05	35.42
74	Y	2.95 ± 0.12	60.1 ± 2.69	6.53 ± 0.18	0 ± 0	0.86 ± 0.43	70.44
75	G	1.18 ± 0.01	27.83 ± 0.15	3.92 ± 0.04	0.96 ± 0.02	2.69 ± 0.02	36.58
76	S	2.79 ± 0.69	51.63 ± 2.38	4.68 ± 0.57	0.46 ± 0.4	2.26 ± 1.05	61.81
77	Y	0.77 ± 0.01	20.65 ± 0.16	2.82 ± 0.01	0 ± 0	0 ± 0	24.24
78	S	3.19 ± 0.26	48.11 ± 2.23	4.34 ± 0.23	0.39 ± 0.34	0.91 ± 0.56	56.93
79	Y	1.5 ± 0.1	41.2 ± 2.31	5.98 ± 0.28	0 ± 0	1.04 ± 0.31	49.72
80	Y	2.38 ± 0.15	42.48 ± 1.47	5.76 ± 0.31	0 ± 0	0.42 ± 0.3	51.04
81	Y	1.64 ± 0.1	37.96 ± 2.51	5.31 ± 0.37	0 ± 0	0.48 ± 0.24	45.4
82	Y	1.49 ± 0.23	38.74 ± 0.57	6.4 ± 0.49	0 ± 0	0 ± 0	46.63
83	Y	1.55 ± 0.15	45.24 ± 1.53	7.36 ± 0.31	0 ± 0	1.18 ± 0.37	55.32

84	S	1.98 ± 0.24	46.01 ± 2.06	4.01 ± 0.24	0 ± 0	1.72 ± 0.98	53.73
85	Y	1.45 ± 0.09	32.71 ± 0.98	4.15 ± 0.17	0 ± 0	0 ± 0	38.3
86	S	2.59 ± 0.31	48.3 ± 3.75	4.12 ± 0.31	0.52 ± 0.45	2.56 ± 1.22	58.1
87	S	2.07 ± 0.19	38.43 ± 3.2	3.51 ± 0.26	0.24 ± 0.21	2.23 ± 0.69	46.49
88	S	2.3 ± 0.12	39.2 ± 0.57	4.53 ± 0.86	0.66 ± 0.38	2.27 ± 0.65	48.97
89	Y	1.52 ± 0.16	42.53 ± 2.38	7.1 ± 0.48	0 ± 0	1.46 ± 0.1	52.62
90	S	2.07 ± 0.25	43.44 ± 2.44	6.35 ± 0.9	0 ± 0	2.1 ± 1.23	53.96
91	S	2.18 ± 0.21	43.89 ± 3	6.39 ± 1.03	0 ± 0	2.05 ± 1.06	54.52
92	G	1.5 ± 0	33.65 ± 0.17	5.11 ± 0.07	1.63 ± 0.01	4.04 ± 0.02	45.93
93	S	3.4 ± 0.48	54.6 ± 0.09	6.24 ± 0.69	0.3 ± 0.26	2.25 ± 1.04	66.8
94	S	1.83 ± 0.18	40.75 ± 1.55	5.91 ± 0.59	0.45 ± 0.39	1.44 ± 0.91	50.37
95	Y	1.57 ± 0.18	42.32 ± 0.87	7.69 ± 0.17	0 ± 0	0 ± 0	51.58
96	S	2.39 ± 0.23	45.55 ± 1.21	6.72 ± 0.81	0.57 ± 0.31	1.95 ± 0.87	57.19
97	G	1.24 ± 0.01	28.34 ± 0.06	4.04 ± 0.02	1.06 ± 0.03	2.96 ± 0.03	37.63
98	S	2.32 ± 0.25	39.3 ± 0.71	5.03 ± 0.64	0.49 ± 0.43	1.76 ± 0.78	48.9
99	Y	1.5 ± 0.21	36.66 ± 1.1	6.25 ± 0.09	0 ± 0	0.23 ± 0.2	44.64
100	S	2.5 ± 0.1	45.89 ± 1.4	5.91 ± 0.18	0 ± 0	0 ± 0	54.3
101	S	2.48 ± 0.14	45.06 ± 1.64	5.28 ± 0.79	0 ± 0	1.84 ± 0.85	54.66
102	Y	0.53 ± 0.01	4.49 ± 0.18	0.62 ± 0.19	0 ± 0	0 ± 0	5.64
103	Y	0.87 ± 0.01	22.62 ± 0.29	2.95 ± 0	0 ± 0	0 ± 0	26.44
104	Y	1.02 ± 0	24.77 ± 0.28	3.3 ± 0.02	0.39 ± 0.11	1.65 ± 0.04	31.12
105	S	1.39 ± 0.01	31.32 ± 0.23	4.7 ± 0.02	1.33 ± 0.01	3.51 ± 0.02	42.25
106	Y	1.32 ± 0.09	37.57 ± 0.37	6.78 ± 0.27	0.34 ± 0.17	1.04 ± 0.32	47.05
107	S	1.76 ± 0.49	36.06 ± 1.78	4.93 ± 0.36	0.43 ± 0.37	1.23 ± 0.79	44.41
108	G	1.91 ± 0.14	42.56 ± 1.6	2.41 ± 0.09	0 ± 0	3.75 ± 0.16	50.64
109	S	3.05 ± 0.2	46.11 ± 1.03	4.5 ± 0.44	0.34 ± 0.17	2.52 ± 0.7	56.52
110	S	2.24 ± 0.25	43.94 ± 2.76	5.4 ± 0.66	0.84 ± 0.34	2.43 ± 0.53	54.85
111	S	1.55 ± 0.18	38.6 ± 1.66	4.62 ± 0.68	0.17 ± 0.15	2.1 ± 0.55	47.05
112	S	1.81 ± 0.16	37.44 ± 2.73	4.24 ± 0.82	0 ± 0	1.81 ± 0.63	45.3
113	G	5.19 ± 1.3	52.31 ± 0.98	3.69 ± 0.09	0 ± 0	6.28 ± 0.11	67.47
114	Y	1.64 ± 0.09	47.33 ± 3.27	7.35 ± 0.43	0.36 ± 0.18	1.8 ± 0.1	58.47
115	G	3.89 ± 1.78	40.6 ± 9.13	2.51 ± 0.55	1.69 ± 1.08	4.85 ± 2	53.53
116	S	1.72 ± 0.13	34.4 ± 2.67	3.96 ± 0.44	0.36 ± 0.31	0.89 ± 0.77	41.34
117	G	1.73 ± 0.02	36.78 ± 0.4	6.83 ± 0.15	2.39 ± 0.04	5.03 ± 0.09	52.75
118	S	2.58 ± 0.2	44.81 ± 1.23	4.89 ± 1.45	0.88 ± 0.6	2.14 ± 0.63	55.3
119	Y	1.44 ± 0.12	37.73 ± 2	4.06 ± 1.3	0.2 ± 0.17	1.57 ± 0.12	45
120	S	2.14 ± 0.1	55.9 ± 2.49	3.77 ± 1.5	0.52 ± 0.18	2.08 ± 0.14	64.41

Table A6. The concentration (μg g^{-1}) of different seed carotenoids including violaxanthin, lutein, zeaxanthin, β-carotene, β-cryptoxanthin, and total carotenoids ± Se measured by HPLC in 120 $F_{2:3}$ chickpea seeds in cross between ICC4475 and CDC Jade grown in greenhouse. Three different cotyledon classes including Y (yellow), G (green) and S (segregating) has shown for each family.

Family	Cotyledon	Violaxanthin	Lutein	Zeaxanthin	β-Cryptoxanthin	β-Carotene	Total
1	Y	1.31 ± 0.2	41.09 ± 2.84	6.41 ± 0.63	0 ± 0	0 ± 0	48.82
2	S	2.18 ± 0.23	40.88 ± 1.71	4.06 ± 0.59	0.54 ± 0.46	1.68 ± 0.83	49.33
3	S	2.66 ± 0.24	46.38 ± 1.52	4.42 ± 0.36	0 ± 0	3.68 ± 0.78	57.13
4	G	1.46 ± 0	32.55 ± 0.18	4.88 ± 0.09	2.07 ± 0.02	3.96 ± 0.01	44.92
5	Y	1.08 ± 0	26.35 ± 0.07	3.67 ± 0.02	1.1 ± 0.02	0.91 ± 0.03	33.11
6	Y	1.19 ± 0	28.31 ± 0.03	3.94 ± 0.01	1.42 ± 0.01	1.9 ± 0.12	36.76
7	S	1.51 ± 0.23	46.83 ± 0.73	6.21 ± 0.4	0.58 ± 0.5	2.16 ± 1.06	57.3
8	S	2 ± 0.21	49.54 ± 3.63	4.86 ± 0.3	0.9 ± 0.61	1.99 ± 1.1	59.29
9	Y	2.89 ± 0.35	41.94 ± 2.51	5.07 ± 0.54	0.49 ± 0.25	0.93 ± 0.47	51.33
10	S	1.89 ± 0.18	38.82 ± 1.08	4.86 ± 0.33	0.48 ± 0.41	0.67 ± 0.58	46.71
11	Y	2.13 ± 0.38	54.48 ± 4.76	6.48 ± 0.72	0.64 ± 0.19	1.24 ± 0.38	64.97
12	S	1.86 ± 0.04	42.16 ± 1.77	3.36 ± 0.33	0 ± 0	2.88 ± 0.43	50.26
13	S	2.62 ± 0.18	39.56 ± 1.11	3.49 ± 0.45	0 ± 0	1.58 ± 0.98	47.25
14	G	1.61 ± 0.01	35.12 ± 0.05	5.82 ± 0.03	2.85 ± 0.04	4.56 ± 0.05	49.96
15	Y	0.99 ± 0	25.47 ± 0.08	3.41 ± 0.01	0.79 ± 0.02	0 ± 0	30.66
16	S	2.1 ± 0.21	50.96 ± 2.86	4.68 ± 0.26	0.61 ± 0.53	1.59 ± 1.09	59.93
17	S	2.34 ± 0.33	56.13 ± 5.09	3.15 ± 0.92	0.36 ± 0.31	3.7 ± 0.95	65.67
18	G	1.26 ± 0.01	28.92 ± 0.09	4.19 ± 0.02	1.57 ± 0.02	2.88 ± 0.03	38.82
19	Y	2.07 ± 0.14	46.92 ± 1.62	4.91 ± 0.41	0 ± 0	1.46 ± 0.51	55.35
20	S	1.91 ± 0.12	42.38 ± 0.15	3.48 ± 0.17	0 ± 0	1.99 ± 0.1	49.76
21	S	2.76 ± 0.93	36.66 ± 2.33	2.54 ± 0.18	0.56 ± 0.48	2.18 ± 0.93	44.7
22	Y	2.89 ± 0.37	44.15 ± 2.44	4.09 ± 0.45	0.76 ± 0.22	1.05 ± 0.43	52.94
23	S	1.89 ± 0.22	46.74 ± 1.45	3.09 ± 0.09	0 ± 0	3.29 ± 1.06	55.01
24	Y	1.79 ± 0.12	50.48 ± 1.63	4.45 ± 0.2	0 ± 0	3.12 ± 0.45	59.83
25	S	3.91 ± 1.3	51.45 ± 2.38	3.69 ± 0.17	0 ± 0	4.2 ± 0.8	63.26
26	G	5.71 ± 1.14	48.99 ± 1.85	4.23 ± 0.67	0 ± 0	5.5 ± 0.47	64.43
27	S	2.48 ± 0.38	49.64 ± 2.59	5.12 ± 0.32	0.71 ± 0.38	1.5 ± 0.71	59.45
28	S	2.63 ± 0.23	51.55 ± 1.27	4.44 ± 0.49	0 ± 0	3.09 ± 0.83	61.71
29	G	7.73 ± 1.65	59.56 ± 3.39	4.86 ± 0.26	4.12 ± 0.33	7.47 ± 1.04	83.74
30	S	3.7 ± 0.18	48.15 ± 1.05	4.15 ± 0.27	0.47 ± 0.41	2.1 ± 0.61	58.57
31	Y	1.92 ± 0.2	41.49 ± 1.96	5.11 ± 0.56	0 ± 0	0.45 ± 0.39	48.96
32	Y	2 ± 0.12	39.38 ± 2.65	4.62 ± 0.53	0 ± 0	1.53 ± 0.49	47.52
33	G	2.24 ± 0.05	40.85 ± 1.29	7.22 ± 0.26	3.57 ± 0.12	5.98 ± 0.09	59.86
34	G	3.15 ± 0.74	38.76 ± 1.5	3.06 ± 0.21	0 ± 0	3.62 ± 0.23	48.59
35	Y	0.89 ± 0.01	23.21 ± 0.05	3.06 ± 0.01	0 ± 0	0 ± 0	27.17
36	Y	0.94 ± 0.01	23.87 ± 0.15	3.2 ± 0.02	0.22 ± 0.11	0 ± 0	28.24
37	G	1.69 ± 0.09	38.75 ± 1.25	2.88 ± 0.07	0 ± 0	2.94 ± 0.11	46.26
38	S	1.96 ± 0.23	44.84 ± 2.63	4.8 ± 0.28	0.65 ± 0.56	1.52 ± 1.31	53.76
39	S	2.78 ± 0.18	43.84 ± 2.15	3.99 ± 0.3	0.78 ± 0.44	2.21 ± 0.76	53.6

40	Y	0.46 ± 0.02	19.02 ± 0.34	2.07 ± 0.08	0 ± 0	0 ± 0	21.55
41	G	1.5 ± 0.01	33.53 ± 0.07	5.28 ± 0.03	2.22 ± 0.05	4.17 ± 0.04	46.7
42	Y	1.13 ± 0.01	26.91 ± 0.15	3.79 ± 0.01	1.28 ± 0.03	1.19 ± 0.09	34.31
43	G	2.02 ± 0.1	38.15 ± 1.06	3.21 ± 0.1	1 ± 0.51	3.1 ± 0.33	47.48
44	S	2.99 ± 0.44	46.9 ± 2.74	4.88 ± 0.4	0.77 ± 0.66	2.5 ± 0.93	58.04
45	S	3.26 ± 0.54	52.72 ± 3.23	5.28 ± 0.88	0 ± 0	2.62 ± 1.12	63.87
46	S	3.5 ± 1.28	54.18 ± 1.74	5.71 ± 0.42	0 ± 0	3.58 ± 1.16	66.97
47	G	3.88 ± 1.21	49.15 ± 1.97	4.19 ± 0.17	0.74 ± 0.64	6.17 ± 0.27	64.13
48	S	2.83 ± 0.27	48.62 ± 4.18	4.5 ± 0.23	0 ± 0	3.11 ± 1.59	59.06
49	Y	2.65 ± 0.28	40.96 ± 2.68	4.76 ± 0.38	0 ± 0	1.41 ± 0.31	49.77
50	S	1.78 ± 0.1	40.67 ± 1.55	5.54 ± 0.79	0 ± 0	1.78 ± 0.84	49.78
51	Y	1.83 ± 0.13	47.54 ± 2.69	5.41 ± 0.3	0 ± 0	2.29 ± 0.37	57.07
52	S	2.69 ± 1.07	37.71 ± 1.62	2.96 ± 0.14	0 ± 0	2.05 ± 0.73	45.41
53	G	1.83 ± 0.09	35.74 ± 0.33	6.05 ± 0.05	3.1 ± 0.05	5.32 ± 0.06	52.03
54	S	2.9 ± 0.75	46.9 ± 3.53	4.41 ± 0.45	0 ± 0	2.01 ± 0.73	56.22
55	Y	1.04 ± 0	25.92 ± 0.06	3.45 ± 0.01	1 ± 0.04	0.56 ± 0.14	31.96
56	S	2.31 ± 0.36	45.35 ± 2.32	4.02 ± 0.54	0 ± 0	3.12 ± 0.94	54.8
57	S	2.99 ± 0.17	40.43 ± 0.71	3.9 ± 0.26	0 ± 0	0.87 ± 0.47	48.18
58	Y	2.52 ± 0.19	41.12 ± 0.88	6.78 ± 0.23	0 ± 0	1.23 ± 0.11	51.65
59	S	2.13 ± 0.42	50.4 ± 1.73	6.56 ± 1.01	0.43 ± 0.38	1.72 ± 1.14	61.25
60	S	1.89 ± 0.37	43.64 ± 1.25	4.66 ± 0.42	0 ± 0	2.05 ± 0.25	52.24
61	G	1.56 ± 0.01	34.32 ± 0.22	5.66 ± 0.05	2.54 ± 0.01	4.37 ± 0.03	48.46
62	S	2.51 ± 0.42	51.4 ± 0.98	5.73 ± 0.37	1.19 ± 0.51	2.53 ± 0.96	63.36
63	Y	3.2 ± 0.35	45.62 ± 1.7	4.52 ± 0.37	0.16 ± 0.14	1.8 ± 0.37	55.3
64	S	2.84 ± 0.42	53.65 ± 1.62	5.44 ± 0.34	0 ± 0	2.93 ± 0.58	64.85
65	S	2.84 ± 1.05	42.35 ± 2.42	4.7 ± 0.52	0 ± 0	2.3 ± 1.08	52.19
66	S	2.38 ± 0.37	41.14 ± 1.4	3.66 ± 0.28	0.48 ± 0.24	3.09 ± 0.59	50.75
67	G	1.29 ± 0	29.44 ± 0.08	4.28 ± 0.02	1.66 ± 0.01	3.12 ± 0.01	39.79
68	S	2.91 ± 0.11	42.87 ± 0.53	3.7 ± 0.15	0.53 ± 0.46	1.75 ± 0.65	51.76
69	S	3.64 ± 1.18	44.08 ± 3.19	3.88 ± 0.17	0.29 ± 0.25	2.95 ± 0.89	54.83
70	Y	0.67 ± 0.02	21.33 ± 0.16	2.75 ± 0.06	0 ± 0	0 ± 0	24.75
71	Y	2.47 ± 0.25	42.45 ± 1.56	7.46 ± 0.47	0 ± 0	1.4 ± 0.28	53.78
72	S	1.79 ± 0.17	42.24 ± 1.94	5.25 ± 0.27	0.75 ± 0.48	1.4 ± 0.75	51.42
73	G	3.2 ± 0.36	49.32 ± 1.73	4.61 ± 0.28	5 ± 0.33	4.45 ± 0.42	66.58
74	Y	2.51 ± 0.31	41.84 ± 1.7	4.78 ± 0.14	0 ± 0	0.75 ± 0.22	49.88
75	S	2.79 ± 0.3	41.43 ± 1.07	3.71 ± 0.29	0 ± 0	1.92 ± 0.74	49.85
76	S	4.3 ± 1.62	48.84 ± 2.23	4.96 ± 0.35	0 ± 0	3.44 ± 0.8	61.54
77	Y	1.68 ± 0.06	48.19 ± 2.03	8.18 ± 0.35	0 ± 0	0.39 ± 0.2	58.44
78	Y	1.42 ± 0.12	40.9 ± 1.48	6.4 ± 0.23	0 ± 0	0.13 ± 0.12	48.85
79	G	6.23 ± 0.48	38.12 ± 2.49	2.53 ± 0.14	0.4 ± 0.34	4.56 ± 0.34	51.84
80	Y	1.4 ± 0.06	38.11 ± 2.15	4.84 ± 0.24	0 ± 0	1.11 ± 0.41	45.47
81	Y	0.92 ± 0.07	26.35 ± 3.32	3.39 ± 0.06	0 ± 0	0 ± 0	30.66
82	S	2.54 ± 0.1	50.32 ± 1.19	3.94 ± 0.27	0 ± 0	2.53 ± 1.15	59.32
83	S	1.69 ± 0.12	46.81 ± 0.8	2.05 ± 0.59	0 ± 0	3.26 ± 0.69	53.81

84	Y	0.98 ± 0	24.86 ± 0.11	3.29 ± 0.02	0.61 ± 0.04	0 ± 0	29.73
85	Y	2 ± 0.09	45.26 ± 2.75	2.82 ± 0.19	0 ± 0	1.77 ± 0.31	51.85
86	Y	1.92 ± 0.13	47.76 ± 1.37	3.85 ± 0.44	0 ± 0	0.95 ± 0.38	54.48
87	G	5.57 ± 1.13	44.06 ± 1.5	0.01 ± 0	0 ± 0	4.5 ± 0.45	54.13
88	G	1.43 ± 0.01	31.72 ± 0.1	4.66 ± 0.01	1.89 ± 0.03	3.75 ± 0.05	43.45
89	G	5.08 ± 1.87	42.25 ± 6.43	2.49 ± 1.28	0 ± 0	5.01 ± 0.93	54.84
90	G	1.37 ± 0	30.42 ± 0.15	4.55 ± 0.02	1.79 ± 0	3.5 ± 0.03	41.63
91	S	1.97 ± 0.19	49.49 ± 2.05	4.56 ± 1.14	0.92 ± 0.8	2.68 ± 0.96	59.63
92	S	1.75 ± 0.38	38.14 ± 8.57	2.3 ± 0.94	0.85 ± 0.53	1.38 ± 0.8	44.41
93	S	1.24 ± 0.08	36.82 ± 1.64	5.51 ± 0.73	0 ± 0	0.38 ± 0.33	43.96
94	Y	1.56 ± 0.11	45.75 ± 0.58	7.16 ± 0.09	0 ± 0	1.15 ± 0.02	55.63
95	G	1.32 ± 0	29.77 ± 0.06	4.43 ± 0.02	1.75 ± 0.01	3.23 ± 0.03	40.5
96	Y	1.06 ± 0.24	37.05 ± 7.85	7.71 ± 1.67	0 ± 0	0.52 ± 0.15	46.34
97	S	3.38 ± 1.55	44.65 ± 1.65	5.56 ± 0.75	0 ± 0	3.25 ± 0.84	56.84
98	S	1.72 ± 0.31	50.26 ± 2.29	6.43 ± 1.01	0.65 ± 0.56	1.2 ± 0.82	60.26
99	S	1.47 ± 0.2	46.11 ± 2.13	5.24 ± 0.35	0.57 ± 0.49	1.04 ± 0.9	54.42
100	S	1.36 ± 0.25	49.54 ± 4.32	6.5 ± 0.15	0 ± 0	0.85 ± 0.09	58.25
101	Y	1.72 ± 0.11	41.92 ± 1.44	6.18 ± 0.37	0 ± 0	1.09 ± 0.14	50.91
102	Y	1.16 ± 0.07	40.53 ± 1.87	4.16 ± 0.44	0 ± 0	0.68 ± 0.11	46.53
103	Y	0.81 ± 0.02	22.36 ± 0.1	2.94 ± 0.03	0 ± 0	0 ± 0	26.11
104	S	2.56 ± 1.11	43.87 ± 2	3 ± 0.87	0.22 ± 0.19	2.74 ± 0.6	52.38
105	Y	1.67 ± 0.18	41.6 ± 1.48	3.07 ± 0.15	0 ± 0	1.34 ± 0.19	47.68
106	S	1.77 ± 0.13	50.72 ± 1.3	3.06 ± 0.88	0 ± 0	3.34 ± 0.56	58.89
107	Y	1.19 ± 0.06	38.4 ± 1.42	2.95 ± 0.21	0.2 ± 0.17	1.98 ± 0.25	44.72
108	Y	1.17 ± 0	27.97 ± 0.1	3.9 ± 0.01	1.37 ± 0	1.53 ± 0.02	35.93
109	S	1.79 ± 0.13	46.54 ± 2.53	4.65 ± 0.41	0.43 ± 0.37	2.38 ± 0.71	55.79
110	S	1.53 ± 0.2	43.05 ± 1.09	4.38 ± 0.78	0.65 ± 0.56	2.63 ± 1.05	52.24
111	Y	1.23 ± 0.04	37.29 ± 0.72	2.69 ± 0.06	0 ± 0	1.13 ± 0.13	42.34
112	S	2.55 ± 1.18	40.69 ± 1.98	4.11 ± 0.45	0 ± 0	3.26 ± 0.8	50.61
113	Y	1.69 ± 0.06	41.22 ± 0.6	5.09 ± 0.27	0 ± 0	2.14 ± 0.11	50.14
114	S	1.25 ± 0.1	37.55 ± 2.53	4.59 ± 0.38	0 ± 0	1.81 ± 0.98	45.2
115	S	2.13 ± 0.83	39.11 ± 1.13	3.73 ± 0.42	0 ± 0	2.38 ± 0.57	47.35
116	G	1.22 ± 0.01	28.63 ± 0.05	4.06 ± 0.02	1.48 ± 0.01	2.63 ± 0.03	38.01
117	Y	1.24 ± 0.04	37.25 ± 0.26	3.98 ± 0.09	0 ± 0	2 ± 0.07	44.48
118	S	1.98 ± 0.79	36.68 ± 2.19	4.28 ± 0.47	0 ± 0	1.73 ± 0.7	44.66
119	Y	1.13 ± 0.06	32.48 ± 1.76	5.83 ± 0.36	0 ± 0	0.28 ± 0.15	39.72
120	S	1.16 ± 0.04	34.64 ± 0.37	4.16 ± 0.32	0 ± 0	0.74 ± 0.06	40.7

Table A7. Name of the genes involved in isoprenoid and carotenoid pathways with their accessions, copy numbers, exons numbers and size in chickpea, Arabidopsis and *Medicago truncatula* collected from NCBI BLAST® online database

	Chickpea			Arabidopsis			Medicago		
Gene	Accession	Exon no	Size (Kbp)	Accession	Exon no	Size (Kbp)	Accession	Exon no	Size (Kbp)
DXS1	LOC101509339	10	5.5	AT3G21500	9	3.9	MTR_4g118640	9	5.7
DXS2	LOC101495911	11	8.3	AT4G15560	9	4.8	MTR_2g020590	10	9.4
DXS3	LOC101501120	10	6	AT5G11380	10	4.5	MTR_8g068265	9	6.4
DXS4	LOC101514041	10	9.2	-	-	-	MTR_3g107740	8	7.9
DXR1	LOC101494630	12	6.1	AT5G62790	13	4.3	MTR_4g106870	12	6.5
DXR2	LOC101490762	13	12	-	-	-	MTR_8g012565	12	7.5
HDR1	LOC101489866	10	4.4	AT4G34350	10	3.4	MTR_4g069030	10	4.7
HDR2	-	-	-	-	-	-	MTR_4g069070	10	5.4
GGPPS1	LOC101509231	1	2.4	AT4G36810	1	1.9	MTR_8g078070	1	2.4
GGPPS2	LOC101490130	1	1.8	AT2G23800	1	1.7	MTR_5g019460	1	1.6
PSY1	LOC101513117	9	5.5	AT5G17230	9	4.3	MTR_5g076620	7	5.6
PSY2	LOC101501341	5	3.5	-	-	-	MTR_5g012060	2	4.7
PSY3	LOC101489077	5	2.5	-	-	-	MTR_5g090780	5	13
PSY4	LOC101509318	6	3	-	-	-	-	-	-
PDS	LOC101499699	13	10	AT4G14210	15	6.6	MTR_5g042243	3	0.94
ZISO1	LOC101501611	4	2.8	AT1G10830	4	2.5	MTR_8g097190	4	4.3
ZISO2	LOC101502677	4	3.2	-	-	-	MTR_3g084950	4	3.6
ZDS	LOC101493508	15	7.6	AT3G04870	14	4.9	MTR_1g081290	14	7.4
CRTISO1	LOC101500336	5	3	AT1G06820	13	4.3	MTR_4g134780	5	3.1
CRTISO2	LOC101494469	13	8.2	AT1G57770	10	4	MTR_1g054965	14	7.7
LCYB	LOC101508316	1	2.5	AT3G10230	2	2.6	MTR_7g090150	1	1.9
LCYE	LOC101509804	11	8.9	AT5G57030	11	3.6	MTR_2g040060	11	9.3
BCH1	LOC101511972	7	3.1	AT4G25700	7	2.6	-	-	-
BCH2	LOC101509699	7	6.1	AT5G52570	7	2.9	MTR_6g048440	7	6.8

CYP97A	LOC101501150	15	8.3	AT1G31800	15	4.3	MTR_7g079440	15	9.3
CYP97B	LOC101502085	15	10	AT4G15110	14	4.3	MTR_5g009110	14	11
CYP97C	LOC101497194	9	7.4	AT3G53130	12	6.2	MTR_1g062190	9	7.9
CCD1	LOC101500703	14	15	AT3G63520	14	4.1	MTR_8g037315	14	8.4
ZEP1	LOC101503567	16	8.6	AT5G67030	16	4.8	MTR_5g017350	15	12
ZEP2	LOC101492490	16	7.9	-	-	-	-	-	-
VDE1	LOC101494261	5	3.9	AT1G08550	7	3.4	MTR_7g116630	6	4.9
VDE2	LOC101512965	3	7.5	AT2G21860	2	2.3	MTR_8g092050	2	4.6
NSY	LOC101501945	1	2.2	AT1G67080	6	2.1	MTR_7g007280	6	4.2

Table A8. List of main domains of important proteins in isoprenoid and carotenoid pathways with their accessions in chickpea collected from NCBI BLAST® online database

Gene	Accession	Super family N treminal	Super family C treminal
DXS1	LOC101509339	Thiamine pyrophosphate	Transketolase
DXS2	LOC101495911	Thiamine pyrophosphate	Transketolase
DXS3	LOC101501120	Thiamine pyrophosphate	Transketolase
DXS4	LOC101514041	Thiamine pyrophosphate	Transketolase
HDR	LOC101489866	4-hydroxy-3-methylbut-2-enyl diphosphate reductase (lytB_ispH)	-
GGPPS1	LOC101509231	Isoprenoid Biosynthesis enzymes-Class 1	-
GGPPS2	LOC101490130	Isoprenoid Biosynthesis enzymes-Class 1	-
PSY1	LOC101513117	Isoprenoid Biosynthesis enzymes-Class 1	-
PSY2	LOC101501341	Isoprenoid Biosynthesis enzymes-Class 1	-
PSY3	LOC101489077	Isoprenoid Biosynthesis enzymes-Class 1	-
PSY4	LOC101509318	Isoprenoid Biosynthesis enzymes-Class 1	-
ZISO1	LOC101501611	NnrU	-
ZISO2	LOC101502677	NnrU	-
DXR1	LOC101494630	NADB_Rossmann	1-deoxy-D-xylulose 5-phosphate reductoisomerase
DXR2	LOC101490762	NADB_Rossmann	1-deoxy-D-xylulose 5-phosphate reductoisomerase
PDS	LOC101499699	NADB_Rossmann	-
ZDS	LOC101493508	NADB_Rossmann	-
CRTISO1	LOC101500336	NADB_Rossmann	-
CRTISO2	LOC101494469	NADB_Rossmann	-
LCYB	LOC101508316	NADB_Rossmann	-
LEC	LOC101509804	NADB_Rossmann	-
ZEP1	LOC101503567	NADB_Rossmann	Forkhead-associated
ZEP2	LOC101492490	NADB_Rossmann	Forkhead-associated
NSY	LOC101501945	NADB_Rossmann	-
BCH1	LOC101511972	Fatty acid hydroxylase	-
BCH2	LOC101509699	Fatty acid hydroxylase	-
CYP97A	LOC101501150	Cytochrome P450	-
CYP97B	LOC101502085	Cytochrome P450	-

CYP97C	LOC101497194	Cytochrome P450	-
CCD1	LOC101500703	Retinal pigment epithelial membrane protein	
VDE1	LOC101494261	Lipocalin / cytosolic fatty-acid binding protein	-
VDE2	LOC101512965	Lipocalin / cytosolic fatty-acid binding protein	-

Table A9. Similarity matrix of DXS1-4 and PSY1-4 in chickpea has shown in the following table using Bio Edit sequence alignment editor software (Hall, 1999).

GENE ID	***DXS2***	***DXS3***	***DXS4***
DXS1	0.842	0.662	0.557
DXS2		0.659	0.535
DXS3			0.528
DXS4			
GENE ID	*PSY2*	*PSY3*	*PSY4*
PSY1	0.635	0.615	0.484
PSY2		0.816	0.527
PSY3			0.542
PSY4			

Table A10. The forward (F) and reverse (R) primer sequences of 19 candidate genes including phytoene synthase (*PSY1*, *PSY2*, *PSY3* and *PSY4*), phytoene desaturase (*PDS*), 15-cis-zeta-carotene isomerase (*ZISO1* and *ZISO2*), ζ-carotene desaturase (*ZDS*), prolycopene isomerase (*CRTISO1* and *CRTISO2*), lycopene β-cyclase (*LCYB*), lycopene ε-cyclase (*LCYE*), β-carotene hydroxylase (*BCH1* and *BCH2*), zeaxanthin epoxidase (*ZEP1* and ZEP2), violaxanthin de-epoxidase (*VDE*), crotenoid 9,10(9',10')-cleavage dioxygenase 1 (*CCD1*) and neoxanthin synthase (*NSY*) involved in the carotenoid biosynthesis and six housekeeping genes including actin 1 (*Act1*), elongation factor 1-alpha (*Ef1α*), glyceraldehyde-3-phosphate dehydrogenase(*GAPDH*), initiation factor 4a (*IF4a*), heat shock protein 90 (*HSP90*) and 18S ribosomal RNA (*18SrRNA*) with their amplicon size are shown in the following table.

Gene name	Primer sequences	Amplicon length (bp)
PSY1	F 5´-GGTGAGTGATTGTGCTCATTTG-3´	112
	R 5´-CCAATCTCGATTCCCACCTATC-3´	
PSY2	F 5´-GCCCAAAGAGAATGCCAAATC-3´	97
	R 5´-CCCACTTCCAAACTTTATCCTAGA-3´	
PSY3	F 5´-CACTTTGTGTATTGCAGTGTGG-3´	76
	R 5´-TGGTGTGATGTGAGAAGCATTA-3´	
PSY4	F 5´-CCTTCCTTCACAAGGCTTATCT-3´	116
	R 5´-GTCTGAACTGTACATGGCCTAA-3´	
PDS	F 5´-GCAGGACTGGCTGGTTTAT-3´	127
	R 5´-ACCAGTCTCCATCTTCATCTTTC-3´	
ZISO1	F 5´-TGGCCGTCAAAGATTACCTAAA-3´	106
	R 5´-CATAAGTGGGTGTGCAAAGTAAG-3´	
ZISO2	F 5´-ATTCCACTGGGTTCGGTAAG-3´	107
	R 5´-ACTGTGGACACCAGCAAATA-3´	
ZDS	F 5´-GGGACCTGGTAAAGATCCATTT-3´	107

	R 5´-CACCTTCCATGCTGTCTATGT-3´	
CRTISO1	F 5´-TGCCGTTGAAGAATGGAAGA-3´	99
	R 5´-ACGCCTAAATCTCCACGAATAG-3´	
CRTISO2	F 5´-TGTTATTGGGTCTGGGATTGG-3´	108
	R 5´-CCAGAACTTCCACCAGGAATAA-3´	
LCYB	F 5´-GCTATTGACCCAAACCCTAGAT-3´	102
	R 5´-CCAAGTTGTGTCAAGGCAATC-3´	
LCYε	F 5´-GTCGACTTACGGTAACGCTATT-3´	100
	R 5´-CTCATCGGCGAAATCCTCTT-3´	
BCH1	F 5´-GAACTCCGAGAGGTTCACTTAC-3´	119
	R 5´-GAACTTCTCCACCCTCCATTT-3´	
BCH2	F 5´-GTCCCATCATAGAGCAAGAGAAG-3´	115
	R 5´-GATGAGTCCCTTGTGGAAGAAA-3´	
ZEP1	F 5´-GATGGTGCCTTCTTCGTAACT-3´	108
	R 5´-GTGGACACGTGCAGGATAAT-3´	
ZEP2	F 5´-GATGATGCACTTGAGCGTACTA-3´	101
	R 5´-GCCTCATCACGACTCAAACA-3´	
VDE	F 5´-GGAGAAGAAGGTAGAGGAAGGA-3´	118
	R 5´-CCCTTCTGCCAACCTTTGTA-3´	
CCD1	F 5´-TGGCTCTGAGGCTGTTTATG-3´	110
	R 5´-GCACGAATGATTTCCCGATATTC-3´	
NSY	F 5´-GCGGAACAAGTTTCCCTTTATG-3´	95
	R 5´-TCATCCAACCACACACCATAG-3´	
Act1	F 5´-GCCTGATGGACAGGTGATCAC-3´	62
	R 5´-GGAACAGGACCTCTGGACATCT-3´	
Ef1α	F 5´-TCCACCACTTGGTCGTTTTG-3´	64
	R 5´-CTTAATGACACCGACAGCAACAG-3´	
GAPDH	F 5´-CCAAGGTCAAGATCGGAATCA-3´	65
	R 5´-CAAAGCCACTCTAGCAACCAAA-3´	
IF4a	F 5´-TGGACCAGAACACTAGGGACATT-3´	60
	R 5´-AAACACGGGAAGACCCAGAA-3´	
HSP90	F 5´-GCAGCATGGCTGGTTACATGT-3´	63
	R 5´-TGATGGGATTCTCAGGGTTGA-3´	
18SrRNA	F 5´-ACGTCCCTGCCCTTTGTACAC-3´	61
	R 5´-CACTTCACCGGACCATTCAAT-3´	

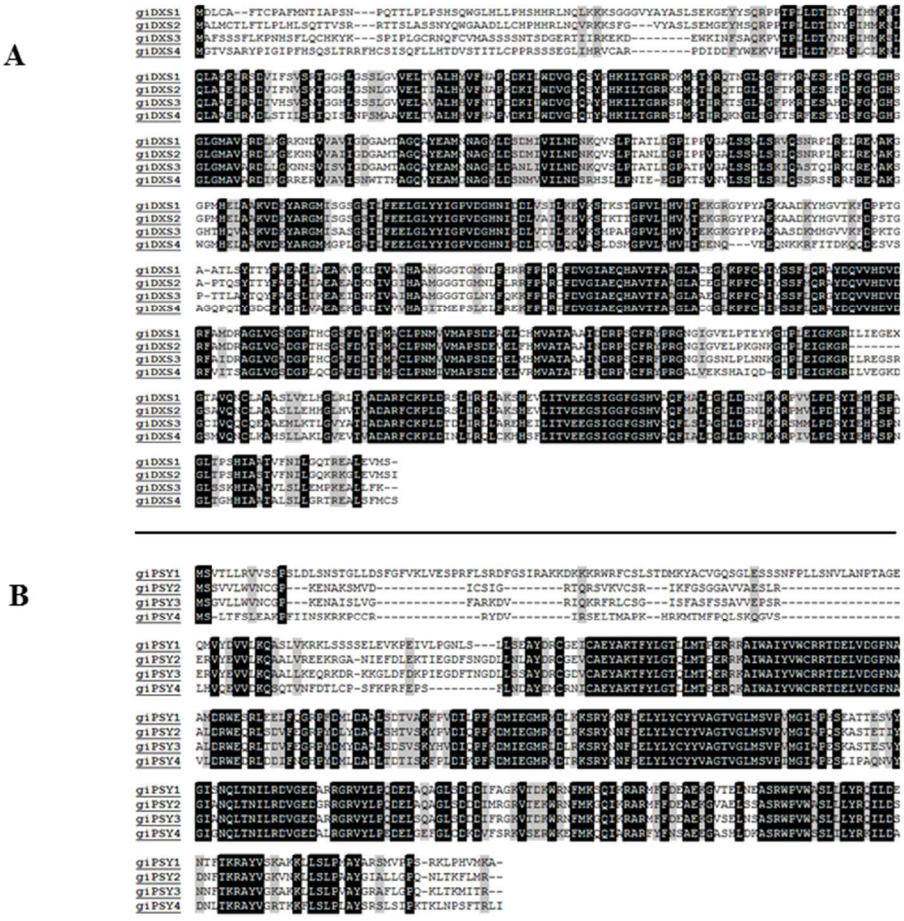
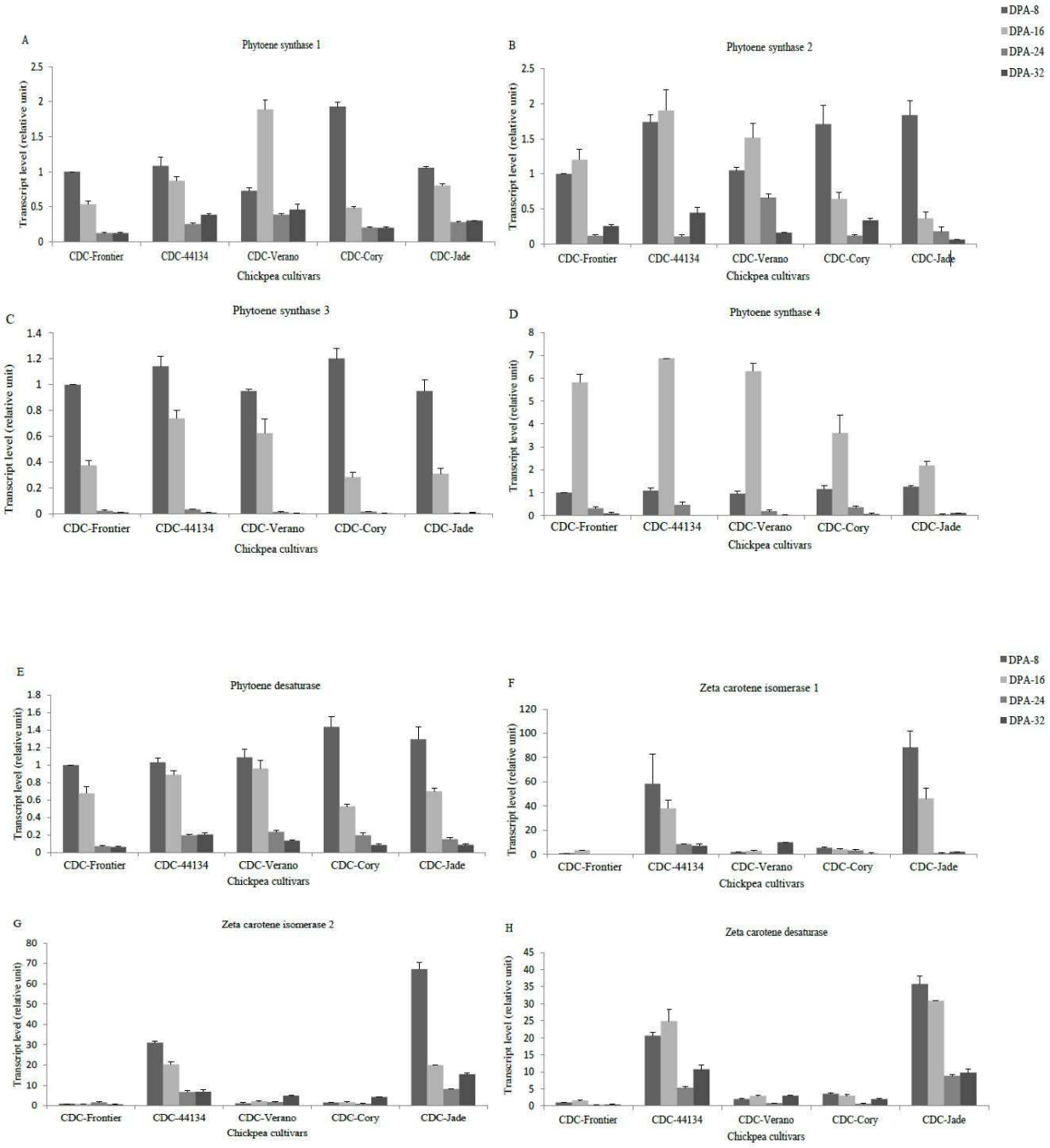
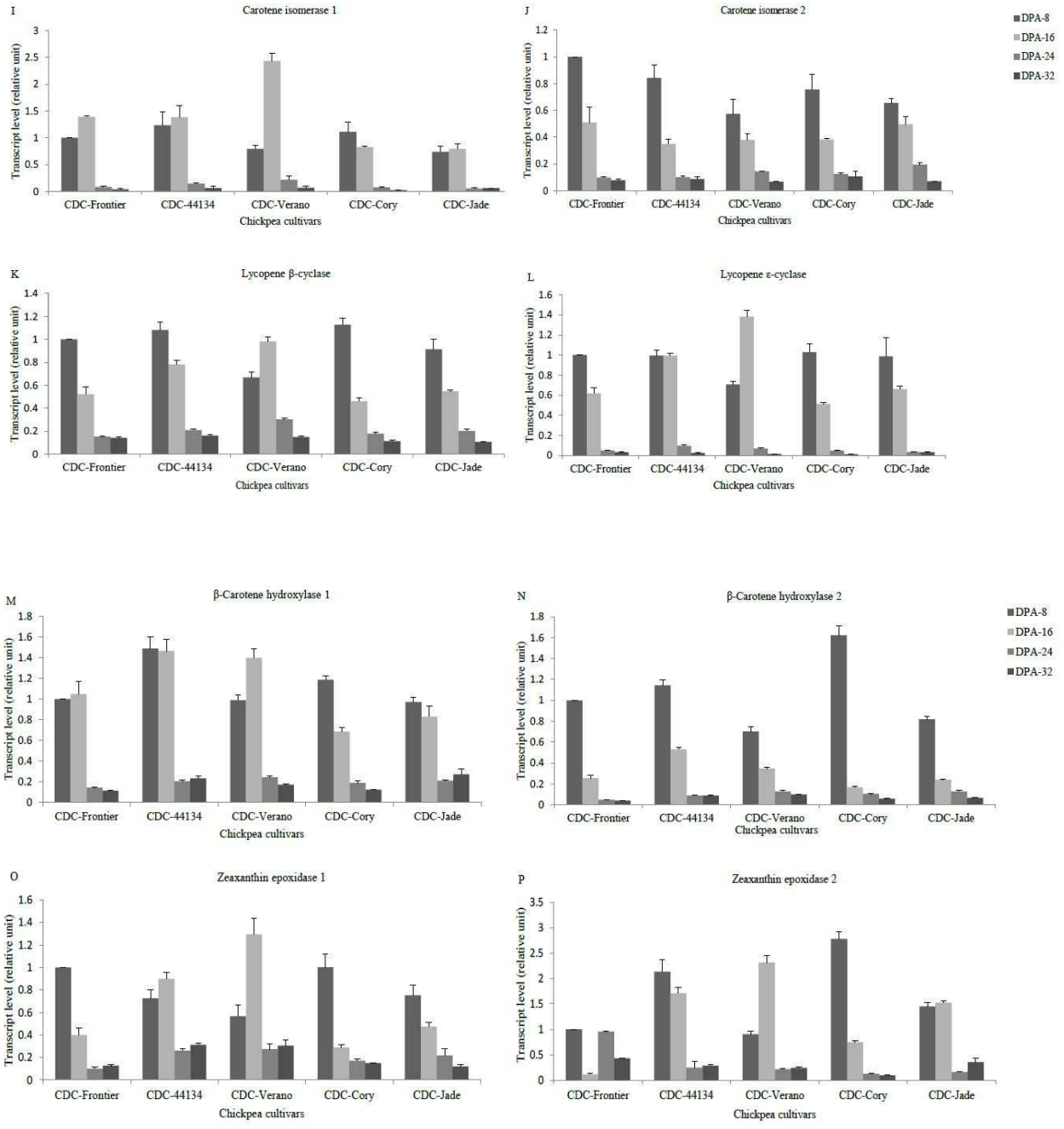


Figure A1. The alignment of protein sequences of DXS1-4 (A) and PSY1-4 (B) in chickpea using Bio Edit sequence alignment editor software (Hall, 1999). Identical and similar amino acid shaded in black and grey respectively.


DPA-8
DPA-16
DPA-24
DPA-32
A
Phytoene synthase 1
B
Phytoene synthase 2
C
Phytoene synthase 3
D
Phytoene synthase 4
E
Phytoene desaturase
F
Zeta carotene isomerase 1
G
Zeta carotene isomerase 2
H
Zeta carotene desaturase
Transcript level (relative unit)
CDC-Frontier
CDC-44134
CDC-Verano
CDC-Cory
CDC-Jade
Chickpea cultivars


I
Carotene isomerase 1
J
Carotene isomerase 2
DPA-8
DPA-16
DPA-24
DPA-32
Transcript level (relative unit)
CDC-Frontier
CDC-44134
CDC-Verano
CDC-Cory
CDC-Jade
Chickpea cultivars
K
Lycopene β-cyclase
L
Lycopene ε-cyclase
M
β-Carotene hydroxylase 1
N
β-Carotene hydroxylase 2
O
Zeaxanthin epoxidase 1
P
Zeaxanthin epoxidase 2

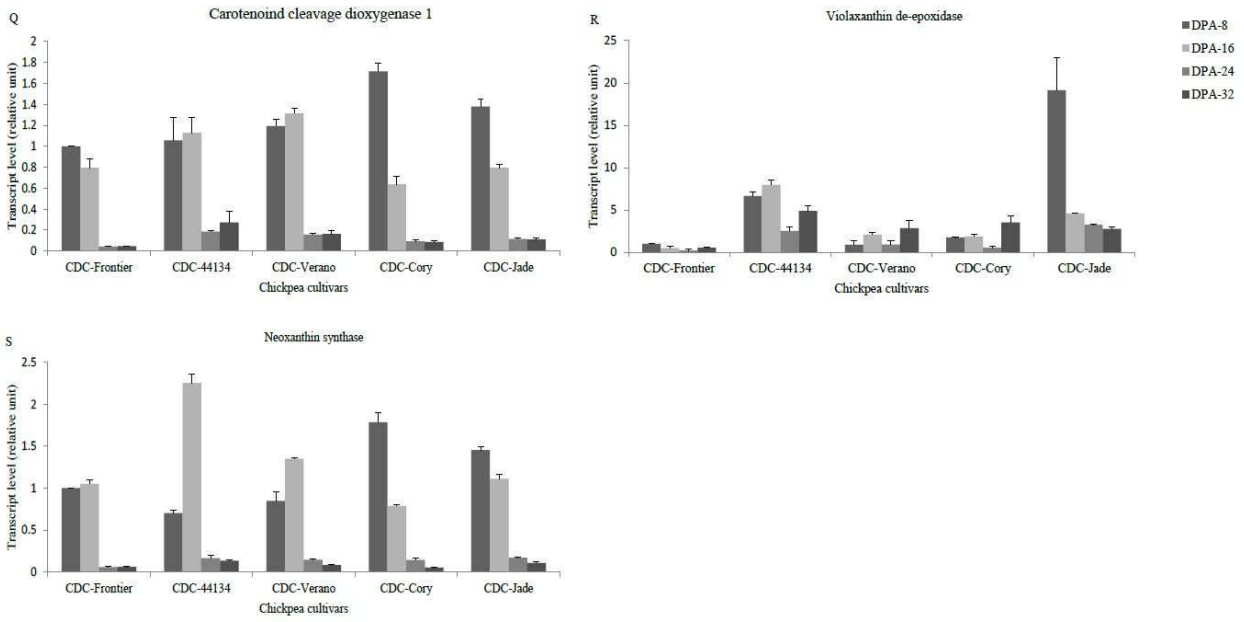


Figure A2. Expression pattern of carotenogenic genes including phytoene synthase 1 (A), phytoene synthase 2 (B), phytoene synthase 3 (C), phytoene synthase 4 (D), phytoene desaturase (E), 15-cis-zeta-carotene isomerase 1 (F), 15-cis-zeta-carotene isomerase 2 (G), ζ-carotene desaturase (H), carotene isomerase 1 (I), carotene isomerase 2 (J), lycopene β-cyclase (K), lycopene ε-cyclase (L), β-carotene hydroxylase 1 (M), β-carotene hydroxylase 2 (N), zeaxanthin epoxidase 1 (O) zeaxanthin epoxidase 2 (P), crotenoid 9,10(9',10')-cleavage dioxygenase 1 (Q) violaxanthin de-epoxidase (R), and neoxanthin synthase (S) in chickpea seeds at four developmental stages 8, 16, 24 and 32 days post-anthesis (DPA) in five cultivars CDC Frontier, CDC 441-34, CDC Verano, CDC Cory and CDC Jade.

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