Translating Biochemistry to Breeding for High Carotenoid Traits in Maize

Catherine Kandianis[1] and Torbert Rocheford[1, 2]

[1]Department of Crop Sciences, University of Illinois
[2]Department of Agronomy, Purdue University

Abstract

Combined use of association mapping and linkage analysis is highly effective in the identification of loci governing quantitatively inherited traits. In this strategy, the underlying genetic basis and function of QTL can be tested through a hypothesis driven approach that draws upon biochemical information from model species. Results from the linkage mapping experiment can then be reconsidered in the context of a larger biochemical system. Understanding of the genetic architecture controlling carotenoid concentrations in maize endosperm has been enhanced by this strategy through the identification of two QTL, LCYε and CrtR-B1 (Harjes, 2008; Yan, 2008) that significantly affect the synthesis and conversion of carotenoids within the pathway. Previous QTL analyses indicated that a locus in bin 9.07 significantly affected carotenoid traits in multiple populations. On the basis of the QTL effect and location, we proposed that genetically controlled degradation processes could be the contributing factor and tested ZmCCD1, a maize homolog of the carotenoid cleavage dioxygenase family. A polymorphism in the promoter of ZmCCD1 was found to associate with changes in total carotenoid concentration as well as lutein. The allelic series discovered in this process was used to design allele-specific markers for use in a linkage mapping analysis of carotenoid QTL in the A619 x SC55 $F_{2:3}$ population. Results indicate that the position of a significant and large effect for lutein and total carotenoid concentrations lies at the ZmCCD1 map location, and reveals that the allele associated with a strong degradation effect is dominant. Interactions of ZmCCD1 with known loci LCYε and CrtR-B1 are evaluated in the context of statistical epistasis and kernel development.

Introduction

Malnutrition has provided a driving force for the development of micronutrient dense staple crop varieties. Enhancement of mineral and nutrient concentrations in crops could be easily, although perhaps incrementally, made by the recombination of superior varieties accompanied by the pursuit of transgressive segregants. To address the need in a reasonable timescale, however, the adoption of a product development approach focused on targeted genetic improvements is required. The approach of biofortification in maize being taken by Harvest Plus, a program established by the Consultative Group on International Agriculture Research (CGIAR), focuses on the improvement of provitamin A, iron and zinc concentrations in grain through conventional plant breeding and modern biotechnology (Pfeiffer, 2007). Thus far, both strategies have made significant improvements in provitamin A concentration by exploiting the genetic and biochemical information known for the carotenoid biosynthesis pathway. Using a similar approach in the United States, breeding programs targeting increased xanthophyll

concentrations have been created to satisfy dietary requirements that aid in the prevention of macular degeneration (Chucair, 2007).

Best known for its role in producing yellow/orange pigment in seed endosperm, carotenoid biosynthesis occurs in all tissues within the maize plant. The functional role of these 40-carbon compounds is perhaps most apparent in the photoprotection of chlorophyllous tissues. However, carotenoids are also present in maize roots, where carotenoid degradation has been implicated as a pathogen signaling mechanism (Sun, 2008), in seed embryo, in which the production of absisic acid from carotenoids is critical for germination (Schwartz, 1997a), and in seed endosperm, where a gain of function mutation in a pathway enzyme resulted in the ability to select for yellow grain (Robertson, 1987). To our knowledge, the biological function of carotenoids has not imposed a selective advantage on the viability of yellow versus white seed, as corn hybrids of both color classes perform well.

Formation of carotenoid pathway precursors competes with the production of chlorophyll (Rodriguez-Concepcion, 2006), but substrate is committed to carotenoid biosynthesis once chemically modified by phytoene synthase, an enzyme encoded by the y1 locus in maize (Buckner, 1996). As indicated in Figure 1, the main branch leading away from phytoene is irreversible and produces several colorless carotenoid precursors including phytofluene, zeta-carotene isomers, and neurosporene isomers (not listed in Figure 1). The production of lycopene marks the first pigmented carotenoid in the pathway, and serves as the main substrate to a bifurcated pathway that leads to the α- and β-carotenoid branches. Shunt of carbon substrate to either branch is controlled by the lycopene cyclases; chemical modification by *lycopene beta cyclase* (LCYβ) is required for both branches, whereas modification by *lycopene epsilon cyclase* (LCYε) only affects the α-branch. Within each branch, carotenoids undergo a series of chemical reductions which transforms the molecules from highly fat soluble carotenes (α-carotene and β-carotene) to the slightly less fat soluble xanthophylls (zeinoxanthin, lutein, β-cryptoxanthin, and zeaxanthin), as depicted in Figure 1. Maize primarily accumulates lutein and zeaxanthin. As compared to other cereal grains, maize has the greatest phenotypic diversity in β-carotene, β-cryptoxanthin and α-carotene content which are all considered provitamin A units (Harjes, 2008).

Characterization of the carotenoid pathway at the DNA sequence and biochemical levels has been achieved using information from plant model species such as Arabidopsis, algae and tomato (DellaPenna, 2006; Matthews, 2007). Investigation of the effect of pathway enzymes on the final carotenoid phenotype has historically been achieved through the use of genetic mutants, in vitro (or recombinant) expression, and biochemical profiling by liquid chromatography that is both technically demanding and costly. Production of transgenic maize for increased provitamin A concentration has leveraged cross-species biochemical information in the metabolic engineering of higher endosperm carotenoid levels (Aluru, 2008). While this method has been successful, lessons from the re-engineering of Golden Rice revealed that endogenous regulation of the pathway can thwart the biosynthetic potential of transgenic events (Paine, 2005). Kernel carotenoid concentrations has been found to be quantitatively inherited in cereal

crops (Wong, 2004; Chander, 2008; Stevens, 2007; Islam, 2004; Salas-Hernandez, 2008), indicating that there could be multiple endogenous control points that transgenic and conventional breeding approaches alike need to address.

The question remains: if DNA sequence or protein homologies exist between biological systems, can a known biochemical framework in model systems be used to help explain QTL in maize? Using the characterized biochemical frameworks from model plant species, Harjes (2008) and colleagues investigated the control of substrate flux through the pathway bifurcation (see Figure 1) which was hypothesized to contribute to the regulation of the β-carotene containing branch. Surveys of a genetically diverse pool of germplasm revealed three significant polymorphisms in the gene encoding LCYε. Strong statistical associations led to the conclusion that genetic variation at this locus significantly altered the ratio of the branches, leading to increased β-carotene. Supporting evidence from an eQTL experiment, carotenoid QTL analysis and a mutagenesis study all pointed to LCYε as the causal factor for the modification of substrate flux. These results allowed the design of PCR-based markers targeted to the three polymorphisms which are currently being used in provitamin A breeding programs. Since report of LCYε as a causal QTL, discovery of *carotenoid reductase B1/ β-carotene hydroxylase* (CrtR-B1), has led to the definition of a second major QTL affecting the conversion of β-carotene to downstream substrates and to the identification of a rare allele contributing to higher provitamin A (Yan, 2008).

Use of the carotenoid trait system has been highly successful in proof of concept experimentation for a multi-faceted approach to discovering the function of biochemical QTL through the use of known biochemistry, QTL mapping and association mapping. Within this system, much remains unknown regarding gene/enzyme interactions and feedback regulation, as well as the unexplored question of carotenoid degradation. Carotenoid concentrations can be dramatically reduced in cereals during grain fill (Kean, 2007) and post-harvest storage (P.Beyer, M.Grusak, personal communications).

We have begun to explore the concept of genetically controlled degradation by maize orthologs of the carotenoid cleavage dioxygenase family (CCD) which have been biochemically characterized in Arabidopsis (Auldridge, 2006). In this species, members of the CCD family differ in substrate preferences; AtCCD1 is reported to reduce β-carotene and lutein seed levels; AtCCD4 has been observed to have an effect on all carotenoid substrates (D.DellaPenna, personal communication); the NCED subfamily has been implicated in the formation of downstream substrate abscisic acid from zeaxanthin (Schwartz, 1997b). Homologs of the CCD family have not been studied to a large extent in maize because of the complexity of the locus (Tan, 2004), the inadequacy of in vitro system expression (Vogel, 2008) and the lack of maize genomic sequence. Molecular characterization of the *White Cap 1* (Wc1) maize mutant, which is associated with a reduction in kernel color, has facilitated cloning of the maize CCD1 locus (Tan, 2004). The combined reports suggest that ZmCCD and AtCCD gene families have similar functions in the removal of carotenoids and, therefore, a specific focus of this research is to determine whether AtCCD1 and ZmCCD1 share substrate specificity for β-carotene.

If so, this would make the ZmCCD1 locus a primary target of selection in maize provitamin A breeding programs.

An integrative approach was taken to determine the in vivo function of ZmCCD1 (herein noted as CCD1), evaluate its map location relative to the bin 9.07 location of the wc1 locus and determine its interaction with loci responsible for the accumulation of carotenoid metabolites. The combined results of association mapping of CCD1, QTL mapping in the A619 x SC55 $F_{2:3}$ population segregating at loci LCYε, CrtR-B1 and CCD1, and metabolic profiling of developing maize endosperm will be discussed. An evaluation of the CCD1 locus in provitamin A and high carotenoid breeding programs will be made.

Materials and Methods

Germplasm Development, Field Evaluation and Sample Collection

A diverse association panel of 282 inbred lines was grown in one-row plots in a randomized alpha(0,1) incomplete block design in Champaign-Urbana, Illinois during the summers of 2002, 2003, 2004, and 2005. The maize association panel was selected as a genotypically diverse germplasm set for which there was no selection of inbreds based on phenotype (Flint-Garcia, 2005; Liu, 2003). Inbreds were selected from temperate, tropical and subtropical backgrounds to maximize allelic diversity within the panel and minimize the LD within lines.

An $F_{2:3}$ population comprising 227 families was derived from parents A619 and SC55, which were selected on the basis of their high total carotenoid and high β-carotene levels, respectively. The population was grown during the summer of 2005 in two environments, the University of Illinois Urbana-Champaign and the International Maize and Wheat Improvement Center (CIMMYT), El Batan, Mexico. The experimental design at both locations was a randomized alpha (0,1) incomplete block design with two replicates. The families were planted in single row plots of 5m rows, with 76 cm between rows. Each plot was thinned to a density of approximately 15 plants per 5m, or 43000 plants ha^{-1}. Seven to nine plants were sib-pollinated within each row, using a plant only once as a pollen source. Seed was bulked within each row after shelling. Seed was stored at room temperature for approximately four months, and then an aliquot of approximately 10g of seed from each row was stored at -80^{o}C until vitamin extraction could be performed.

Maize inbreds A619, SC55, CI7, DE3, KUI3 and B77 were planted in blocks based on desired genotypic contrasts of LCYε, CrtR-B1 and CCD1. The five contrasting blocks were: (1) A619, SC55; (2) KUI3, SC55; (3) CI7, DE3; (4) CI7, KUI3; (5) KUI3, B77. Within each block the inbreds were planted in two-row plots. The experiment was grown in a randomized split plot design with three replicates in Champaign-Urbana, Illinois during the summer of 2008. In each block, pollinations were made to obtain selfed ears of each parental inbred (P1 and P2), ears of the hybrid P1 x P2, and ears of the reciprocal cross P2 x P1. Developing ears were harvested on the basis of days after pollination (DAP) according to a 15 point time-course including 12, 14, 16, 18, 21, 24,

27, 30, 33, 35, 38, 40, 45 DAP, a fresh final harvest point (55-65 DAP) and a final harvest point with heat treatment of approximately 100 degrees F in a dryer for five days. Whole ears were flash frozen in liquid nitrogen and stored at -80 C. Frozen kernels were removed and divided into four portions to 1) stage the reproductive maturity of the kernels (R1 through R4), 2) obtain fresh and dry five-kernel weights (3 replicates), 3) measure kernel volume (7 replicates), and 4) reserve a bulk seed sample for carotenoid and transcript profiling. Only carotenoid profiles from the self-pollinated inbreds will be discussed.

Carotenoid Extraction and Quantification

Germplasm for the association panel and A619 x SC55 $F_{2:3}$ progeny were phenotyped for carotenoid concentrations by High Performance Liquid Chromatography described in Harjes (2008). Concentrations were represented on a dry weight basis (micrograms carotenoid per gram kernel dry weight).

Fresh frozen bulk kernel samples from the 2008 planting were used for carotenoid profiling. Embryos from 4-6 kernels were removed, and the remaining frozen endosperm was coarsely homogenized. Further homogenization was performed on 30-50 mg (WW) of sample using a QIAGEN Tissuelyser II homogenizer and 4 mm steel ball bearings. Carotenoids were extracted in microtubes using 600 µl of 2:1 methanol:chloroform containing BHT (1 mg/mL) and tocol as an internal standard by further homogenization. After addition of 400 µl water and 200 chloroform, the samples were vortexed for 15 minutes and spun at 12 000 g for 10 minutes. The bottom fraction was collected, dried, and resuspended in 200 µl injection buffer (95:5 acetonitrile: ethyl acetate). HPLC analysis was carried out on 50 µl of the final extract.

Carotenoids were separated by HPLC on a C18 column (Spherisorb ODS2 5 micron, 150 × 2.1 mm, Column Engineering, Ontario) with a Shimadzu LC-20AD HPLC at variable flow rates with solvent A (acetonitrile: water [9:1 v/v]) and solvent B (ethyl acetate) and the following gradient: 0–20 min, 5% to 77% B, 1.0 mL/min; 20-20.2 min, 77 to 100% B, 1.0 mL/min; 20.2-22.2 min, 100% B, 1.5 mL/min, 22.2-22.4 min, 100-5% B, 1.5 mL/min; 22.4-25 min, 5% B, 1.0 mL/min. HPLC peak areas were integrated at 450 nm.

Genotypic Data Collection and Molecular Marker Design

DNA sample collection and PCR-based genotyping for the A619 x SC55 $F_{2:3}$ families are described in Stevens (2007). A total of 114 microsatellite markers were assayed, which was done in-kind by Pioneer. Of these markers, 101 are publicly available on MaizeGDB. The remaining 13 herein assigned a “pio” prefix are proprietary Pioneer markers.

Three markers specific to maize genes *LCYe* (LCYε-MZA), *CrtR-B1 (*HYDb1-D4-ds) and *CCD1* (CCD1-pro) were used to genotype the population. Marker LCYε-MZA was designed to distinguish a three-nucleotide difference in exon 1 of LCYε between A619 and SC55 (*LYCe-MZA-P1-L(SC55)*, ATT TTT CTG GTA TTT ATT CAG C; *LYCe-MZA-P2(A619)*, AAG GCT ACT ACC TCC ATG AAA; *LYCe-MZA-All-R1*,

AAT GAG AAT AGT ATG AGA TCG). This was accomplished using inbred-specific sequence kindly provided by Pioneer. Marker HYDb1-D4-ds, designed by Dr. Debra Skinner, detects a 12 bp indel segregating between the inbreds (*HYDb1-D4-F2*, ACC GTC ACG TGC TTC GTG CC; *HYDb1-D4-R1*, CTT CCG CGC CTC CTT CTC). Marker CCD1-pro was designed to distinguish three allelic states of the ZmCCD1 promoter in the association panel (allelic classes being B73-like, Wc and wild type or WT); inbreds A619 and SC55 differed in the alleles of the promoter polymorphism, allowing this marker to be used for both the association analyses and QTL analyses The ZmCCD1 promoter marker was designed to have a conserved right primer and allele-specific left primer to enable detection of heterozygotes, and a Wc and B73-like chimera arising from intragenic recombination within a highly mutable locus (Tan, 2004). Primers in the four-marker system used to detect the allelic series of CCD1-pro are as follows: *CCD1-WT-L1*, ACT CAC TCG TAC TCA TCT ATC CAA; *CCD1-WC-L1*, CCG TGC TCG GAC AGA ATA GT; *CCD1-B73-rev-L1*, CTC ACA CGT GTC AAC GCC; *CCD1-ALL-R1*, GTC GTT TCG GTG GCT GTC.

Genetic Map Construction and Composite Interval Mapping

Linkage maps were generated using JoinMap® Version 3 as in Stevens (2007). The marker order is consistent with the physical map locations of the SSR/ PCR primers found in the publicly available maize sequence. Comprised of the 117 markers described above, the total map length is 1727.8 cM, with an average of 16.1 cM between markers.

Direct estimates of individual carotenoid pool concentrations were obtained. Derived traits expressed as ratios or sums of the direct estimates were calculated. The derived traits more accurately describe the genetic effects on product-substrate conversion, competition between pathway branches or metabolites, and changes in pools with similar chemical structures. Best linear unbiased predictors of all direct and derived traits were generated for the two replicates of the Mexico location of the QTL mapping experiment. Phenotypic data for the Illinois location was not included in this analysis. QTL mapping was done by composite interval mapping (CIM) using stepwise regression for cofactor regression with PLABQTL software (Utz and Melchinger, 2003). This software is based on the Haley-Knott regression method (Haley, 1992) and permits the evaluation of models varying in gene action. Regression models are evaluated on the basis of model fit criterion such as AIC and adj R^2. For the A619 x SC55 population, an additive model produced an adequate fit to the data. Most epistatic effects between detected QTL for most traits were negligible. A threshold corresponding to an experiment-wise Type I error rate of α=0.25 was used for QTL selection of each trait (approximately LOD 2.9). This error rate has been accepted as a suitable genome-wide threshold for exploratory QTL analyses (Utz and Melchinger, 2003). Trait variation was modeled with single and all two and three factor interactions of known genes LCYε, CrtR-B1 and CCD1 with Proc GLM (SAS). A Bonferroni corrected experiment wise error rate (α=0.01) was used to test significance for model components. Contrasts and least square means were drawn to evaluate gene action models for LCYε, CrtR-B1 and CCD1 when they were found to be associated with a given trait effect.

Statistical Analyses

Association analysis was conducted using a general linear model incorporating population structure as implemented in TASSEL (www.maizegenetics.net; Bradbury, 2007). This approach accounts for the multiple levels of relatedness based on random genetic markers that are used to establish population structure. Using a general linear model, a statistical association between trait variation and ZmCCD1 marker genotype was evaluated using the following linear model:

$y_i = u + x_i + \mathrm{B}_1(x_{i1} - \overline{x_{.1}}) + \mathrm{B}_2(x_{i2} - \overline{x_{.2}}) + e_i$ where y_i is the carotenoid concentration for a given trait, μ is the pedigree/line mean, x_i is the effect of marker genotype of the ith allele, $\mathrm{B}_1(x_{i1} - \overline{x_{.1}})$ is the coefficient of linear regression of y_i on population structure covariate 1 (non-stiff stalk), $\mathrm{B}_2(x_{i2} - \overline{x_{.2}})$ is the coefficient of linear regression of y_{ij} on population structure covariate 2 (stiff stalk), and e_i is the random experimental error. Three allelic classes were tested in the marker genotype term: B73-like, WT (wild type) and Wc (white cap). A fourth class of B73-like + Wc was collapsed into the Wc class, as no significant difference was found between these two class means. Pedigrees that were considered "white" were excluded from the analysis. Missing marker data also limited the number of individuals that were used in the single-gene analysis (year 2001, n=29; year 2002, n=40; year 2003, n=77; year 2005, n=98), reducing the power of analyses. Therefore, a single-year analysis was run only for the year 2005. In addition, a combined analysis including all four years was run, using the best linear unbiased predictors (BLUPs) as the trait means. An experiment-wise type I error rate α=0.05 cutoff was used for significance.

Results

Comparison of Trait Distributions for Germplasm

To reach the objectives of our investigation, two different maize populations were selected. The association mapping panel contains a genetically diverse germplasm set that minimizes linkage disequilibrium but maximizes phenotypic diversity, whereas the A619 x SC55 $F_{2:3}$ mapping population has less phenotypic variability but maintains family structure and enables linkage analysis. Comparisons of the carotenoid profiles between the A619 x SC55 $F_{2:3}$ mapping population and the maize association panel are shown in Table 1. White lines (those with little or no carotenoid pigment) are included in the phenotypic distribution of the association panel. No lines from the mapping population were white. Greater variation for most traits except β-carotene and the colorless carotenoids, phytoene and phytofluene, was observed in the association panel in comparison to the mapping population. This was consistent with the greater genetic diversity of the association panel, but also shows that genetic variation in A619 and SC55 affecting β-carotene and the colorless carotenoids provided complementary levels of variation in the $F_{2:3}$ progeny. The means for most traits in the association panel were higher than or equal to those of the mapping population. Since A619 was selected as a high total carotenoid parent, we expected that it would contribute to a higher progeny mean. It is possible that the A619 contribution was affected by deleterious SC55 alleles. The mean for β-carotene was found to be higher in the mapping population than in the

association panel, which follows prediction based on the parental lines having been selected primarily for high β-carotene levels.

Characterization of Association Panel for ZmCCD1 Promoter Allelic Frequencies

Previous study of ZmCCD1 (indicated that a wild type (WT) version of the locus is most commonly found in teosinte species. Two other alleles, B73-like and Wc (in reference to the classical *white cap* locus), found in diverse maize lines are presumed to be orthologs of WT. These two alleles are each marked by a distinct and different transposable element insertion immediately 5' to the start site (Figure 2). In the case of Wc, this insertion is accompanied by a tandem duplication of 12-24 copies of CCD1. (Tan, 2004; D. McCarty, personal communication). The B73-like allele was most prevalent in the association panel, with a frequency of 0.57 as opposed to the Wc class with a frequency of 0.24 (Figure 2). White germplasm had higher frequency of the Wc allele, suggesting that the creation of many white inbreds was accompanied by selection of not only y1 but also Wc. Presence of Wc, however, does not completely remove carotenoids from maize endosperm as there are more than a dozen yellow lines with the Wc allele.

Association Analyses for ZmCCD1

Statistical associations between the allelic classes of the CCD1 promoter with all absolute and derived traits were examined to determine if the polymorphism was linked to a reduction in carotenoid levels consistent with the proposed function of CCD1 in Arabidopsis (Schwartz, 2001). Using a model accounting for population structure and marker genotype, and applying an experiment-wise type I error rate of α=0.05, the 2005 analysis indicated that allelic differences in ZmCCD1 explain significant variation in lutein (p=0.006) and are marginally significant for total colored carotenoid (p=0.07), which is a trait derived from the summation of the xanthophylls and carotenes (Table 2). No significant effect of marker class was found for β-carotene, or for any of the other absolute or derived traits. A combined year analysis indicated the marker polymorphism to account for considerable variation in lutein (p=0.0076) as well as derived traits for total colored carotenoid (p=0.0036), β-carotene/total (p=0.0115), α-carotene/lutein (p=0.0025) and β-carotene/zeaxanthin (p=0.0035). Marginal associations were found for zeaxanthin (p=0.067) and lutein/total (p=0.069) at an α=0.05 cutoff. On average, the Wc allele results in a 35% decrease in lutein for the combined analysis, and a 40% reduction in the year 2005 analysis; decrease of total colored carotenoid is largely due to the reduction in lutein as judged by the trait distributions (Table 1). Effects seen for derived traits which utilize total carotenoid concentration in the ratio may be attributed to fluctuations in the large proportion of lutein that is typically present in the total carotenoid amount.

Detection of QTL in the A619 x SC55 F2:3 Population

Significant QTL for 8 direct and 4 derived traits were found in this population (Table 3). A range of 3-6 significant loci per trait explaining a large proportion of the variation for most traits (adj R^2=0.37-0.59) were found using additive models. Significant QTL were found on all chromosomes. QTL commonly appeared to affect

more than one trait within the same branch or between parallel branches in a pattern that was consistent with enzyme specificity in the carotenoid biosynthesis pathway.

β-branch carotenoids, consisting of β-carotene, β-cryptoxanthin and zeaxanthin, were affected by QTL on chromosome 10 within the umc1506 - CrtR-B1 – umc1993 interval with intra-interval marker positions of 79 cM, 93 cM and 104 cM, respectively. The QTL positions for each of the three traits were at 94 cM, 88 cM, and 88 cM, respectively. As previously mentioned, the CrtR-B1 enzyme controls conversion of β-carotene to β-cryptoxanthin and β-cryptoxanthin to zeaxanthin (Yan, 2008). Additive effects estimates indicate that the SC55 allele at this locus is correlated with an increase in β-carotene (0.37 ug), and a decrease in downstream β-cryptoxanthin (-0.20 ug) and zeaxanthin (-0.50 ug) substrates. A similar result was observed for the β-carotene/β-cryptoxanthin ratio (2.74 ug). This is independent confirmation of the importance of the CrtR-B1 locus as a target for enhancing β-carotene accumulation. A similar chemical conversion could be catalyzed by a reductase enzyme like CrtR-B1 in the α-branch (consisting of α-carotene, zeinoxanthin and lutein). This QTL did not have a significant effect on the α-branch carotenoids individually, but the results indicated that CrtR-B1 accounts for a significant amount of variation in the α-carotene/ zeinoxanthin ratio (12.2%, 0.08 ug).

For both α-branch and β-branch carotenoids, a QTL was detected on chromosome 9 in an interval flanked by CCD1. The SC55 allele exhibits a pronounced negative effect on lutein (reduction of 1.79 ug), in addition to reductions in zeaxanthin (-0.86 ug), β-cryptoxanthin (-0.11 ug) and zeinoxanthin (-0.07 ug). The QTL accounted for as much as 29% of the variation in individual xanthophyll compounds and contributed a 40-60% reduction in the lutein and zeaxanthin.

Control of the branch bifurcation by LCYε was also evident in this population. Approximately 15% of the variation for the derived trait α / β branch was explained by this locus (position of QTL effect = 92 cM; position of LCYε = 91). LCYε was also found to explain a large proportion of the variation for lutein (11.3%).

The QTL analysis also revealed several loci with large and pleiotropic effects that have not yet been verified by candidate genes (Figure 1). Source substrates phytoene and phytofluene appeared to be co-regulated by QTL in bins 1.11, 7.02 and 9.08. The A619 chromosomal segment at QTL in bins 1.11 and 7.02 was associated with a dramatic increase in both. QTL affecting carotene concentration appeared in bins 5.02 and 5.03. Contribution from SC55 for this locus appears to account for the same amount of variation (13.1%, 0.38 ug) in β-carotene as did CrtR-B1 (10.0%, 0.37 ug) within this population.

Allelic Interactions Within Selected Loci

Additive and dominance effects models were applied to the LCYε, CrtR-B1 and CCD1 loci. Only traits significantly affected by these genes were examined. Linear and quadratic contrasts were calculated using Proc GLM to assess the significance of additive

and dominance gene action within loci. LCYε and CrtR-B1 were found to act only in an additive manner on lutein and total carotenoids, where the linear component was found to be significant at a type I error cutoff of α=0.05 (Table 4). CCD1 exhibited significant dominance as well as additive effects as seen by the linear and quadratic components for lutein, zeaxanthin and total carotenoid. Inspection of least squares means by marker class suggested that the SC55 allele for CCD1 is dominant to the A619 allele. This agrees with mutant studies of white cap (P.Stinard, personal communication), where the mutant condition (Wc) is typically found to be dominant.

Digenic Interactions Between Selected Loci

Least squares means analysis was used to examine epistatic interactions between LCYε, CrtR-B1 and CCD1 loci (Figure 3). Due to missing marker data for the CCD1 locus, the LCYε-SC55: CCD1-A619 haplotype class was completely absent and prevented the appropriate contrasts to be drawn for evaluation of an epistatic interaction. For lutein and total colored carotenoid concentration, the LCYε-A619: CCD1-SC55 haplotype class least squares mean was less than that of the low parent value. In a biochemical context the resultant negative epistatic interaction can be explained by the fact that the LCYε-A619 allele has the effect of shunting substrate to the β-branch, away from the α-branch, and CCD1-SC55 allele has the effect of simultaneously degrading the reduced lutein pool. It is hypothesized that the epistatic interaction of LCYε-A619: CCD1-SC55 in reducing the lutein pool would not be evident in the absent haplotype class of LCYε–SC55: CCD1-A619 as this allelic combination would result in more equal provision of substrate to both branches. No combinations of CrtR-B1 and CCD1 yield a significant epistatic interaction for zeaxanthin concentration.

Carotenoid Production During Kernel Development

To validate significant QTL effects, an experiment was initiated in which inbred lines with contrasting LCYε, CrtR-B1 and CCD1 haplotypes were evaluated for carotenoid production potential during kernel development. The effect of degradation during synthesis and conversion was of particular interest, as selection for lines with potential for enhanced accumulation could be confounded by degradation activity of CCD1. Therefore, a profile of carotenogenesis throughout kernel development was performed to provide information related to: (1) temporal characterization of the phases of synthesis, conversion and degradation; (2) haplotype specific effects on coordinated accumulation and removal of carotenoids.

The carotenoid profile from 12 DAP to harvest (65 DAP) indicated that carotenoid production and accumulation are highly dependant upon the haplotype of the line (Figure 4). Inbreds in this experiment were selected to contrast known CCD1 promoter allelic states, where the B73-like allele represents a weak, less active degradation enzyme, and the WC allele represents a strong, overactive version of CCD1. Depicted in Figure 4 are two B73-like lines (CI7 and KUI3) and one Wc line (SC55). Developmental profiles of these lines revealed a significant decrease in lutein for the Wc line, but not the B73-like lines. Profiles of zeaxanthin provided evidence of the Wc allelic effect as well. To our knowledge, this is first in vivo evidence of CCD1 substrate

specificity in maize and provides a biochemical validation of the QTL mapping results. Analysis of the genotype by timepoint interactions is being explored.

Total carotenoid concentration continually increased over kernel development. A rapid rate of carotenoid accumulation occurred from 12 to 27 DAP followed by a slower increase after 27 DAP. Genotypic differences are apparent in total carotenoid profiles as well as those for individual carotenoids. This is in stark contrast to carotenogenesis during the maturation process of sorghum grain where degradation gradually out-competes synthesis, leaving little carotenoid accumulation at harvest (Kean, 2007). Genetically controlled carotenoid synthesis and degradation mechanisms in maize may differ substantially from those in sorghum.

Discussion

This study identified a new candidate QTL, ZmCCD1, which maps to a location in bin 9.07 previously observed to have large effects on multiple carotenoids in a number of mapping experiments (Wong, 2004; Islam, 2004; Stevens, 2007; Chander, 2008). Segregation for CCD1 was found to account for significant phenotypic variation in total colored carotenoid concentration likely through a significant decrease in lutein, with smaller effects on zeaxanthin, zeinoxanthin, and β-cryptoxanthin. These results are in agreement with in vitro experimentation testing the substrate specificity of ZmCCD1, where β-cryptoxanthin and zeaxanthin were found to decrease in the presence of the enzyme (Vogel, 2008), an analysis of the lutein substrate was not possible with the published recombinant system. Therefore this is the first report of lutein as a substrate of ZmCCD1. Considering that a biochemical homology was assumed, the data does not agree with AtCCD1 substrate specificity, which was shown to use β-carotene as a substrate. Of all cleavage dioxygenases in Arabidopsis, AtCCD1 is most similar to ZmCCD1 in amino acid identity (76.55% identical), making the difference in substrate specificity a somewhat unexpected finding. Comparison of LSmeans for the allelic variants of the ZmCCD1 promoter indicated that the Wc allele had a strong degradation effect. Dominance of the strong Wc allele as well as negative epistatic interactions between CCD1 and LCYε revealed that a single copy of Wc could substantially reduce total carotenoids. The profiling results suggested that the decreases in total carotenoids correlated with the Wc-CCD1 genotype are largely attributed to a removal of lutein. The effect of this degradation is most severe around 30 DAP suggesting that there is a temporal separation between peak synthesis and degradation.

In addition to the effects found for LCYε, CCD1 and CrtR-B1, three other QTL each exhibited effects on more than one trait. These QTL showed effects on traits within the same pathway branch or on chemically similar traits in α- and β- branches. The pattern of effects appeared to be consistent with that of an enzyme functioning in the carotenoid biosynthesis pathway. The QTL in bin 5.02/5.03 had an effect on β-carotene, α-carotene and zeinoxanthin. Lycopene beta cyclase (LCYβ), an enzyme encoded by the ps1/vp7 (pink scutellum, viviparous 7) locus, influences the shunt of upstream pathway substrates to both α and β branches (Singh, 2003). The LCYβ locus has been mapped to bin 5.02 (T. Brutnell, personal communication). LCYβ is therefore a plausible candidate

on the basis of both map location and biochemical function. Zeta-carotene desaturase (ZDS/ Vp9), involved in the conversion of colorless substrates downstream of phytofluene (Li, 2007), has been associated with significant carotenoid trait variation in several QTL mapping populations. The map position of this gene in bin 7.02, and its biochemical function also leads to speculation that the ZDS locus could be the QTL affecting phytoene and phytofluene. Additionally, a QTL in bin 1.11 for both phytoene and phytofluene with no known (or obvious) testable biochemical homolog was detected. Its position in contig ctg64 of the published maize sequence should accommodate in silico queries to yield a list of plausible candidates.

In addition to being an excellent model system for biochemical genetics, the carotenoid pathway in cereals provides a source of precursor vitamins and antioxidants that are limiting in most diets. A major goal of the Harvest Plus micronutrient biofortification program is to achieve higher concentrations of provitamin A, elevated levels of β-carotene, α-carotene, and β-cryptoxanthin. Intensive phenotypic evaluation of germplasm pools have been essential to the increases made in the program. With the application of allele-specific molecular markers designed to identify favorable (and often rare) genotypes, the breeding process has been simplified and some selection can be performed prior to planting. Currently, the Rocheford Laboratory is combining this allele-specific marker assisted strategy for LCYε and CrtR-B1 with single kernel genotyping (Gao, 2008) to increase the frequency of favorable alleles in breeding populations. Researchers at CIMMYT are following coordinated parallel and complementary strategies.

Development of high total carotenoid populations can also benefit from a marker assisted selection strategy for CCD1. High total carotenoid breeding can be simplified through the visual selection of more yellow/ orange kernel pigmentation since there is a high correlation between total carotenoid and kernel color within breeding populations. Previous experimentation has shown that the effect from bin 9.07 reduces kernel color (Stevens, 2007); based on results from this study, it is likely that CCD1 is responsible for this effect. High carotenoid breeding programs in developing countries have crossed donor lines with enhanced orange pigment to predominantly white Mexican and African maize varieties in an effort to combine the alleles for higher total carotenoid with favorable agronomic traits (K. Pixley, personal communication). If the frequencies of the Wc-CCD1 allele in the Mexican and African populations are similar to the white germplasm in the maize diversity association panel, the recurrent parents may harbor deleterious alleles at both CCD1 and y1 which will dilute the high carotenoid effect upon recombination. Therefore, use of markers for CCD1 alleles should be useful to enhance recovery of the desirable donor genetics while excluding heritable degradation effects.

The genetic architecture of the carotenoid trait system appears to be predominated by biochemical QTL that explain much of the trait variation and exhibit pleiotropic effects. Association mapping can be quite useful in cases where candidate gene prediction is desired for QTL with large trait effects, as in the case for those loci in bins 5.02/5.03 and 7.02. Statistical associations between quantitatively inherited traits and causal loci have been performed for many biochemical, regulatory and developmental

genes with traits including starch composition/ quality (Wilson, 2004), plant and inflorescence architecture (Weber, 2007), and flowering time (Salvi, 2007). It is critical to note that each report selected candidate loci prior to conducting the study, meaning that some prior knowledge of the biology affecting the trait was required. In cases where the candidates cannot be postulated beforehand (as may be the case for some of the carotenoid QTL with smaller effects), genome-wide association mapping will be of use. This method enables investigators to evaluate loci that would not otherwise have a biological basis for testing by using high throughput genotyping methods to maximize the detection of polymorphic sites in the genome (Buckler, 2007). This method of causal QTL discovery works jointly with a unique maize germplasm resource called the Nested Association Mapping (NAM) population. NAM has been developed to take advantage of allelic diversity in the association panel, in combination with controlled population structure from linkage mapping populations (Doebley, 2005). The pairing of association analysis and linkage mapping is proving to yield complementary results, particularly for QTL validation (Ducrocq, 2008; Harjes, 2008), and should be able to further the value of biochemical QTL as targeted breeding tools.

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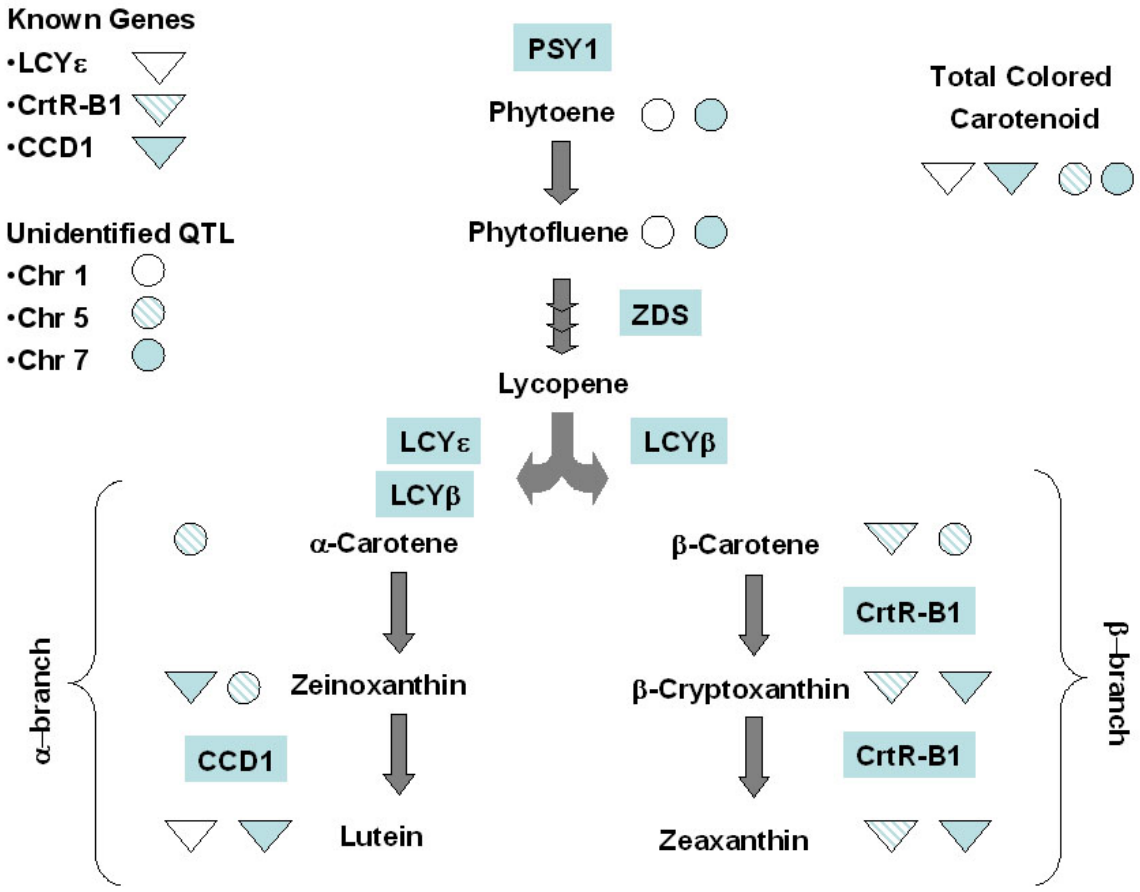


Figure 1: Carotenoid biosynthetic pathway and pleiotropic effects

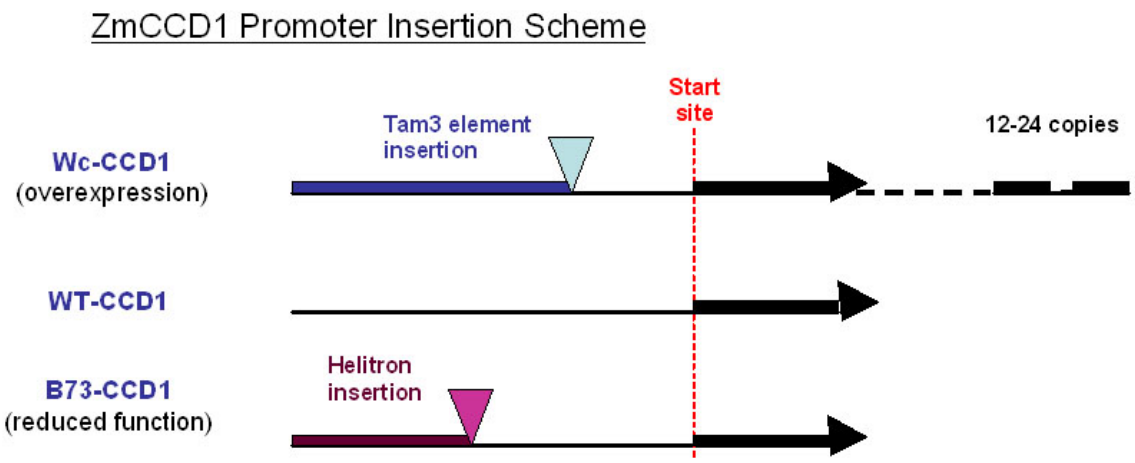
A619 x SC55 F2:3 Population
(Combined Replicates)

Carotenoid Traits	n=227 Mean	Std Dev	Max	Min	Population Parents A619	SC55
Lutein	4.07	2.58	14.31	0.58	6.90	0.38
Zeaxanthin	1.37	1.40	8.06	0.16	5.78	0.29
Zeinoxanthin	0.92	0.49	3.78	0.25	0.94	0.90
β-cryptoxanthin	0.73	0.34	2.08	0.17	1.12	0.28
α-carotene	0.34	0.12	0.82	0.16	3.54	0.38
β-carotene	4.94	1.17	9.48	2.86	0.21	3.09
Total Colored	12.38	4.39	32.39	5.54	18.48	5.33
Phytoene	4.74	2.87	14.57	0.36	4.29	0.39
Phytofluene	1.35	0.61	3.37	0.14	1.57	0.16

Association Mapping Panel: 2005
n=220

Carotenoid Traits	Mean	StdDev	Max	Min
Lutein	5.27	3.68	18.66	0.13
Zeaxanthin	4.07	3.74	25.24	0.05
Zeinoxanthin	1.00	1.10	9.12	0.00
β−cryptoxanthin	0.66	0.65	4.18	0.00
α−carotene	0.62	0.78	4.66	0.00
β-carotene	0.51	0.69	3.72	0.00
Total Colored	12.18	7.70	40.59	0.22
Phytoene	1.13	1.46	8.14	0.00
Phytofluene	0.29	0.32	1.57	0.00

Table 1: Trait distributions (μg/ g carotenoid) in association mapping panel and A619 x SC55 $F_{2:3}$ mapping population



	All lines with data		White lines excluded		White lines only	
Genotype	Number	Frequency	Number	Frequency	Number	Frequency
CCD1-B73	103	0.57	92	0.79	11	0.26
CCD1-WT	8	0.04	6	0.05	2	0.05
CCD1-B73+Wc	5	0.03	1	0.01	4	0.10
CCD1-Wc	43	0.24	18	0.15	25	0.60
total individuals	159		117		42	

Figure 2: ZmCCD1 promoter polymorphism and allele frequencies in association panel

	Combined Years n=241			Year 2005 n=98		
Carotenoid Trait	p_Mkr	Rsq_Model	Rsq_Marker	p_Mkr	Rsq_Model	Rsq_Marker
Lutein	0.0076	0.906	0.0549	0.006	0.2134	0.094
Zeaxanthin	0.067	0.9306	0.0343			
Total Colored	0.0036	0.9258	0.0661	0.0709	0.0887	0.0691
Bcarotene/Total	0.0115	0.9458	0.0684			
Lutein/Total	0.069	0.9643	0.0326			
Acarotene/Lutein	0.0025	0.7973	0.0721			
Bcarotene/Zeaxanthin	0.0035	0.6879	0.0664			

Adjusted Means: Combined Years	Lutein	Zeaxanthin	Total Colored Carotenoid	Bcarotene/ Total	Lutein/ Total	Acarotene/ Lutein	Bcarotene/ Zeaxanthin
CCD1-B73	10.31	6.14	19.60	0.07	0.52	0.06	0.08
CCD1-WT	10.79	4.21	17.26	0.09	0.53	0.07	-0.09
CCD1-Wc	6.41	4.06	13.00	0.12	0.43	0.11	1.38

Adjusted Means: Year 2005

	Lutein	Zeaxanthin
CCD1-B73	7.09	4.90
CCD1-WT	5.51	3.58
CCD1-Wc	4.25	4.08

Table 2: Association analyses for ZmCCD1 promoter in 2005 and combined year trait panel using GLM

				Phytoene			**Phytofluene**			**Total Colored Carotenoids**		
Chr.	Chr. Bin	Contig (ctg)	Interval	LOD	Add. (ug g^{-1})	R^2 (%)	LOD	Add. (ug g^{-1})	R^2 (%)	LOD	Add. (ug g^{-1})	R^2 (%)
1	1.11	64	umc2242-umc1979	3.00	-0.49	3.1	3.84	-0.16	7.8			
3	3.07	142	pio_5-umc1489				5.18	-0.16	8			
4	-		pio_7-pio_8							3.46	0.97	7.9
5	5.03	212/217	umc2035-umc2295							4.02	1.07	9.5
7	7.02	297	umc1068-bnlg1094	9.41	-1.41	22.6	17.99	-0.36	34.5	3.04	-0.85	6
	7.04	323/325	umc1944-umc1125	5.32	0.84	9						
8	8.05	354	LCYe-umc1340							3.87	0.98	8.9
9	9.07	391	CCD1-zct128							21.21	-3.65	53.1
	9.08	391	zct128-umc1505	10.08	-1.35	19.8	9.61	-0.24	18.4			
						37.5			**46.6**			**55.6**

				β-Carotene			**β-Cryptoxanthin**			**Zeaxanthin**		
Chr.	Chr. Bin	Contig (ctg)	Interval	LOD	Add. (ug g^{-1})	R^2 (%)	LOD	Add. (ug g^{-1})	R^2 (%)	LOD	Add. (ug g^{-1})	R^2 (%)
2	2.04	77	umc1541-pio_4				6.07	-0.11	10.1			
	2.05	90	pio_4-umc1459							6.61	-0.64	17.4
	2.07	91/105	bnlg1396-dupssr25	5.95	0.34	7.0						
4	4.02	156	pio_08-phi295450	6.29	0.39	9.6						
	4.03	158/176	adh2-umc1142	4.34	-0.27	4.3						
5	5.03	212/217	umc2035-umc2295	10.03	0.38	13.1						
6	6.05	285/287	umc1805-umc1859	4.99	0.30	7.5						
7	7.00	-	umc1241-umc1068				3.52	-0.08	6.7			
9	9.07	391	CCD1-zct128				4.08	-0.11	12.4	4.22	-0.86	29.6
10	10.03	400	umc2017-pio_14				3.19	-0.06	2.5			
	10.05	414	umc1506-CrtR-B1				9.93	-0.20	17.6	4.32	-0.50	9.7
	10.06	417	CrtR-B1-umc1993	6.42	0.37	10.0						
						37.1			**45.5**			**44.1**

				α-Carotene			Zeinoxanthin			Lutein		
Chr.	Chr. Bin	Contig (ctg)	Interval	LOD	Add. (ug g^{-1})	R^2 (%)	LOD	Add. (ug g^{-1})	R^2 (%)	LOD	Add. (ug g^{-1})	R^2 (%)
1	1.03	-	phi339017-pio_1				8.18	0.20	13			
3	3.06	146/147	bnlg2241-pio_5	3.10	-0.01	1.2						
4	-	-	pio_7-pio_8	5.88	0.03	11.9						
5	5.02	208/212	umc1587-umc2035	5.30	0.03	8.3						
	5.03	212/217	umc2035-umc2295				8.78	0.17	12.8			
6	6.05	283	pio_10-umc1114							2.63	-0.55	6.9
8	8.04	-	umc1343-LCYe	5.69	0.03	9.5						
	8.05	354	LCYe-umc1340							5.47	0.64	11.3
9	9.06	389	pio_13-umc1675							4.17	-0.76	7.4
	9.07	391	CCD1-zct128				3.12	-0.07	2.2	12.85	-1.79	28.8
						21.6			**26.3**			**59.2**

				α: β branch			β-Carotene: β-Cryptoxanthin			α-Carotene: Zeinoxanthin		
Chr.	Chr. Bin	Contig (ctg)	Interval	LOD	Add. (ug g^{-1})	R^2 (%)	LOD	Add. (ug g^{-1})	R^2 (%)	LOD	Add. (ug g^{-1})	R^2 (%)
1	1.03	-	phi339017-pio_1							10.90	-0.09	18.0
2	2.05	77	umc1541-pio_4	2.88	0.08	7.5						
6	6.01	259/270	umc1883-umc1186				2.92	-0.78	2.6			
	6.05	283	pio_10-umc1114	4.26	-0.11	16.6						
7	7.04	323	pio_11-umc1944							4.42	0.05	7.9
8	8.05	354	LCYe-umc1340	6.54	0.09	14.6						
9	9.07	391	CCD1-zct128	11.41	-0.21	40.1						
10	10.05	414	umc1506-CrtR-B1				10.36	2.25	3.4			
	10.06	417	CrtR-B1-umc1993				7.63	2.74	4.8	4.53	0.08	12.2
						48.0			**41.8**			**27.8**

Table 3: QTL detected in combined analysis of A619 x SC55 $F_{2:3}$ population, Mexico environment

Trait	Gene	Regression	Pr > F
Lutein	LCYE	linear	0.002
	LCYE	quadratic	0.2519
	CCD1	linear	<.0001
	CCD1	quadratic	0.0001
Zeaxanthin	CrtR-B1	linear	0.0072
	CrtR-B1	quadratic	0.1034
	CCD1	linear	0.0112
	CCD1	quadratic	0.0275
B-Carotene	CrtR-B1	linear	0.0077
	CrtR-B1	quadratic	0.2434
Total Carotenoid	LCYE	linear	0.012
	LCYE	quadratic	0.4572
	CCD1	linear	<.0001
	CCD1	quadratic	<.0001

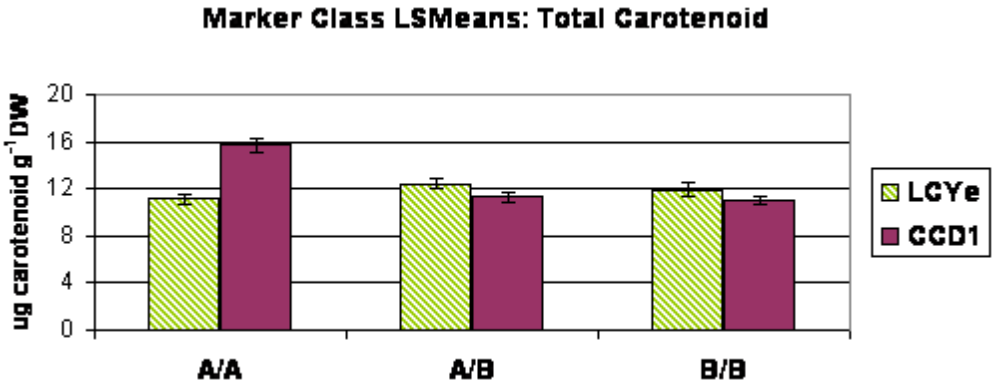


Table 4: Contrasts testing additive and dominant effects for genes LCYε, CCD1 and CrtR-B1. Representation of allelic contribution is: A619 = A, SC55 = B.

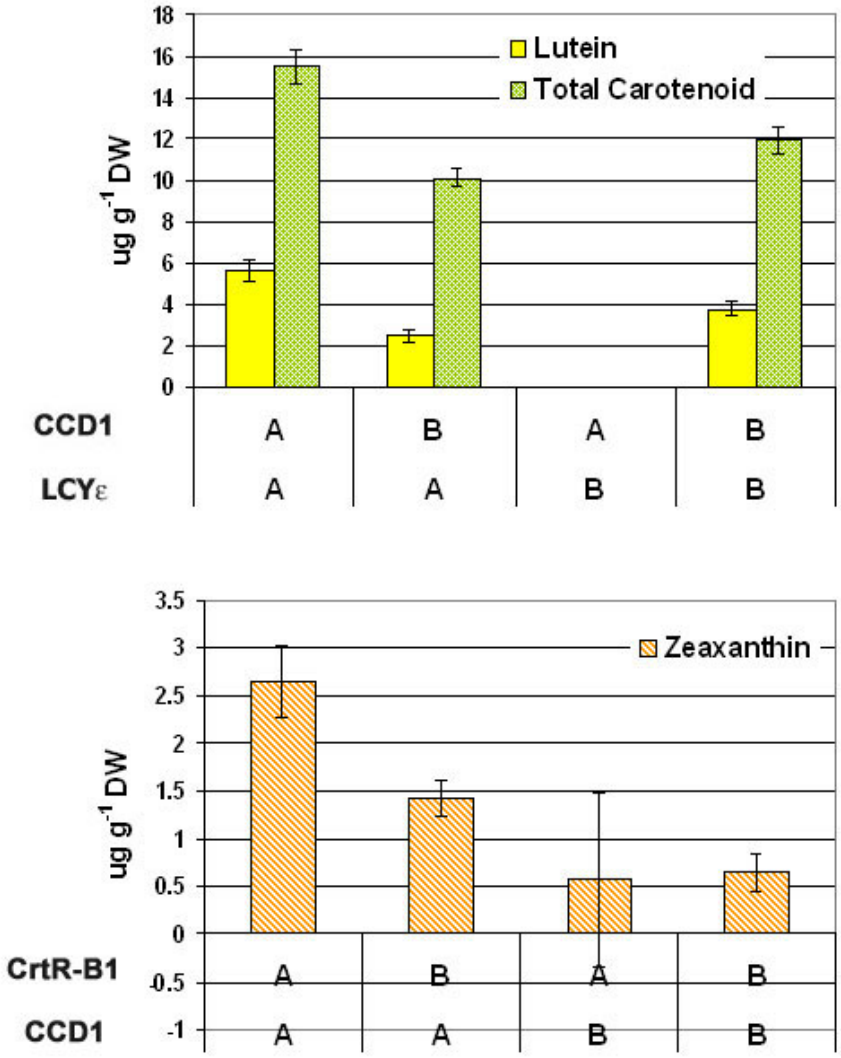
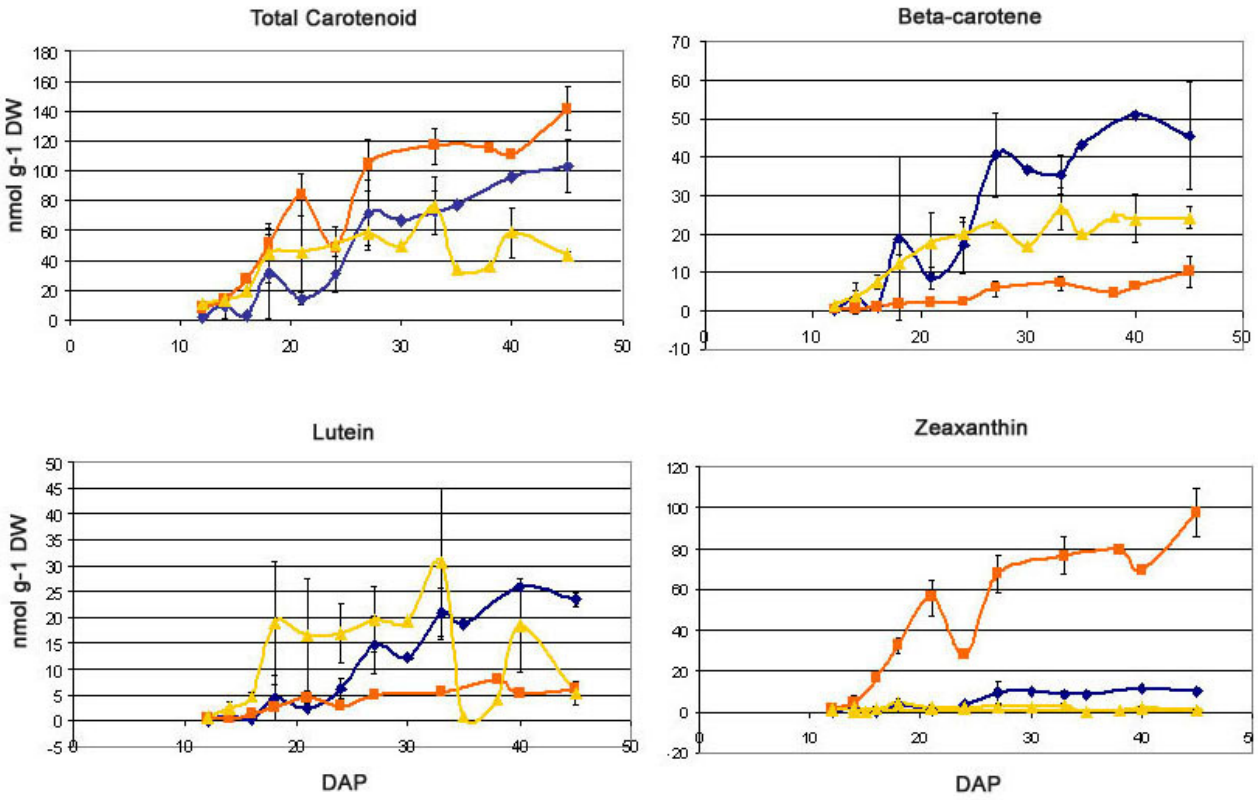


Figure 3: Digenic interactions between selected QTL illustrated through least squares means. Representation of allelic contribution is: A619 = A, SC55 = B.



	LCYε	CrtR-B1	CCD1
CI7	.	weak allele	weak allele
KUI3	weak allele	.	weak allele
SC55	.	weak allele	.

Figure 4: Carotenogenesis profile of selected traits for genotypes varying in QTL haplotypes