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ORIGINAL PAPER

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QTL and candidate genes phytoene synthase and ζ-carotene desaturase associated with the accumulation of carotenoids in maize

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Abstract Carotenoids are a class of fat-soluble antioxidant vitamin compounds present in maize (*Zea mays* L.) that may provide health benefits to animals or humans. Four carotenoid compounds are predominant in maize grain: β-carotene, β-cryptoxanthin, zeaxanthin, and lutein. Although β-carotene has the highest pro-vitamin A activity, it is present in a relatively low concentration in maize kernels. We set out to identify quantitative trait loci (QTL) affecting carotenoid accumulation in maize kernels. Two sets of segregating families were evaluated—a set of $F_{2:3}$ lines derived from a cross of W64a x A632, and their testcross progeny with AE335. Molecular markers were evaluated on the $F_{2:3}$ lines and a genetic linkage map created. High-performance liquid chromatography was performed to measure β-carotene, β-cryptoxanthin, zeaxanthin, and lutein on both sets of materials. Composite interval mapping identified chromosome regions with QTL for one or more individual carotenoids in the per se and testcross progenies. Notably QTL in the per se population map to regions with candidate genes, *yellow 1* and *viviparous 9*, which may be responsible for quantitative variation in carotenoids. The *yellow 1* gene maps to chromosome six and is associated with phytoene synthase, the enzyme catalyzing the first dedicated step in the carotenoid biosynthetic pathway. The *viviparous 9* gene maps to chromosome seven and is associated with ζ-carotene desaturase, an enzyme catalyzing an early step in the carotenoid biosynthetic pathway. If the QTL identified in this study are confirmed, particularly those associated with candidates genes, they could be used in an efficient marker-assisted selection program to facilitate increasing levels of carotenoids in maize grain.

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Introduction

Carotenoids are a class of compounds produced by most photosynthetic organisms that have been shown to be beneficial to both plants and animals. More than 600 different naturally occurring carotenoids have been identified (Britton 1995). Carotenoids are responsible for red, orange, and yellow colors present in some flowers and fruits, with the color of the specific carotenoid determined by the number and location of the double bonds present within the structure (Watson 1962). There are two distinct classes of carotenoids—carotenes, which contain only carbon and hydrogen, and xanthophylls, which contain oxygen groups (Van den Berg et al. 2000). Carotenoids are lipophilic and are found in hydrophobic areas of cells in close proximity to proteins and lipids (Britton 1995; Van den Berg et al. 2000).

Carotenoids serve several purposes in plants. They are essential in photosynthesis, acting both in light collection and photo-protection. They also serve in a non-photosynthetic role by contributing to flower and fruit color as well as being part of the pathway for the biosynthesis of abscisic acid (ABA), an essential plant hormone involved in kernel dormancy. In contrast, the role of carotenoids in animals is not well-defined. Carotenoids have been shown to have antioxidant activity in vitro, but the effects in vivo are not well understood (Van den Berg et al. 2000). With respect to animal health the most prominent function of carotenoids is as pro-vitamin A (Food and Nutrition Board and Institute of Medicine 2000). Several carotenoids have pro-vitamin A activity due to the presence of a vitamin A structure that is part of the overall carotenoid

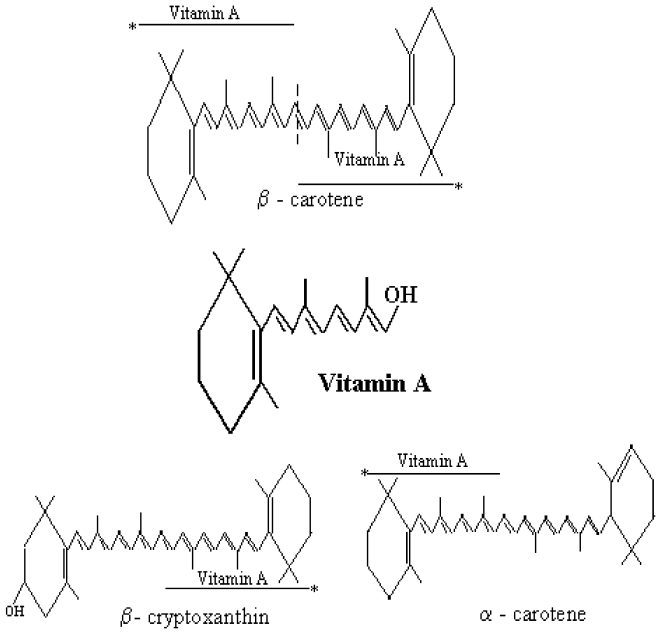

Fig. 1 The chemical structure of vitamin A and carotenoids with pro-vitamin A activity: *β*-carotene, *α*-carotene, and *β*-cryptoxanthin

compound. Vitamin A is released upon digestion of pro-vitamin A by animals (Fig. 1).

An adequate intake of carotenoids with pro-vitamin A activity has been shown to reduce vitamin A deficiency in humans. Such a deficiency is associated with susceptibility to infection, night blindness, rough and scaly skin, and diminished teeth and bone development. Low blood plasma levels of carotenoids have been associated with an increased risk of several degenerative diseases. However, some studies using high doses of synthetic *β*-carotene supplements have shown an increased risk of susceptibility to some diseases, not a decrease. This suggests food sources containing natural carotenoids may be more beneficial than vitamin supplements (Van den Berg et al. 2000).

There are possible economic benefits to animal production systems associated with carotenoids. Levels of *β*-carotene may work synergistically with other fat-soluble vitamins in the protection of broiler leg meat from oxidation (Ruiz et al. 1999). High levels of carotenoid pigments in diets of chickens have also been associated with a desirable color of the egg yolks and of the skin of broilers and fryers (Blessin et al. 1963b; Perez-Vendrell et al. 2001). This coloration of yolks and skin is perceived as associated with good health and quality by some consumers (Hadden et al. 1999).

Yellow maize kernels contain several carotenoid isoforms, including two carotenes—*α*-carotene and *β*-carotene—and three xanthophylls—*β*-cryptoxanthin, zeaxanthin, and lutein (Fig. 1) (Watson 1962; Weber 1987a). Of these two carotenes, *β*-carotene is present in the highest concentration, while either lutein or zeaxanthin is the most prevalent form of the xanthophylls. The

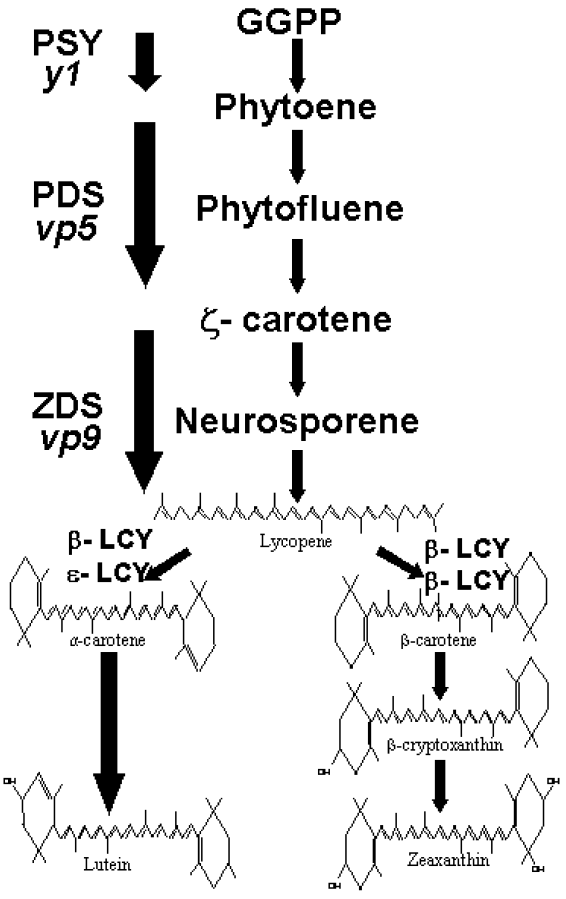

Fig. 2 Biosynthetic pathway for carotenoids, including the cloned genes in maize, encoding enzymes phytoene synthase (*PSY*), phytoene desaturase (*PDS*), and zeta-carotene desaturase (*ZDS*). These enzymes have been associated with mutants of maize *yellow 1* (*y1*), *viviparous 5* (*vp5*), and *viviparous 9* (*vp9*), respectively

horny endosperm contains 74–86% of the carotenoids, floury endosperm has 9–23%, and the rest is present in the germ and bran of the kernel (Blessin et al. 1963a). Substantial variation in the levels of specific forms and in total levels of carotenoids has been shown (Weber 1987b). Moderate to high heritability estimates indicate that breeding for increased levels of both carotenes and xanthophylls should be feasible (Brunson and Quackenbush 1962; Blessin et al. 1963b).

While the carotenoid biosynthetic pathway (Fig. 2) is well characterized in several organisms (Sandmann 1991), in maize it is not yet fully characterized because some of the genes encoding certain enzymes still need to be identified. Characterization of the carotenoid biosynthetic pathway in maize has been facilitated by the analysis of mutants associated with reduced levels of carotenoids. Three genes controlling early steps in the carotenoid pathway have been cloned in maize. The use of these cloned genes as probes on a mapping population will enable the candidate gene approach (Pflieger et al. 2001) to be used for studying the genetic control of quantitative variation in carotenoids.

Studies in several different plant species have examined the relationship between candidate genes and quantitative variation (McMullen et al. 1998; Faris et al. 1999; Prioul et al. 1999; Thorup et al. 2000). Faris et al. (1999) suggested that if the candidate gene can be validated, then it can be used as an efficient molecular marker to aid in selecting desirable alleles. One problem with the candidate gene approach is that it is not known initially if the candidate gene is affecting the trait or if the effect is from another gene linked to the candidate gene. The effect of structural loci in the carotenoid biosynthetic pathway of the Solanaceae on fruit color is of particular relevance to our study (Thorup et al. 2000). Several of the carotenoid structural genes used as probes accounted for a significant portion of the quantitative variation for color within the populations, suggesting that allelic variation at these structural loci may contribute to some of the variation.

The *y1* mutant is a white endosperm mutant with greatly reduced levels of kernel carotenoids. *y1* has been cloned and maps to bin 6.02 in maize (Buckner et al. 1990). Buckner et al. (1996) demonstrated a relationship between the *y1* gene and phytoene synthase. This enzyme is involved in the first dedicated step of carotenoid biosynthesis—the conversion of two molecules of geranylgeranyl pyrophosphate to phytoene. Phytoene desaturase is the second enzyme in the carotenoid biosynthetic pathway and is responsible for a two-step desaturation, taking phytoene to zeta (ζ)-carotene. Phytoene desaturase (PDS) has been associated with the mutant *viviparous 5* (*vp5*), a white endosperm mutant deficient in both carotenoids and ABA. The *vp5* gene has been cloned and mapped to bin 1.02 in maize (Li et al. 1996; Hable et al. 1998). ζ-carotene desaturase (ZDS) is the third enzyme in the carotenoid biosynthetic pathway and is responsible for a two-step saturation from ζ-carotene to lycopene. It has been associated with *viviparous 9* (*vp9*), another white endosperm mutant of maize. A cDNA encoding PDS has been cloned and mapped to bin 7.02 in maize (Luo and Wurtzel 1999; Matthews et al. 2003). Other important genes in the carotenoid biosynthetic pathway of maize still need to be cloned and made available, most notably lycopene β-cyclase, and ε-cyclase, which convert the straight-chain lycopene into β- and α-carotene (Cunningham et al. 1996) by adding two β-rings to β-carotene, and one each of an ε– and β-ring to α-carotene.

The objective of the research reported here was to identify quantitative trait loci (QTL) and to estimate their effects on levels of individual carotenoids and total carotenoids in kernels of a maize population. The mapping of QTL with effects on carotenoid biosynthesis, including QTL in regions of candidate genes, should enable a better understanding of the genetic control of levels of carotenoids in maize kernels.

Materials and methods

Genetic materials

Maize inbreds W64a and A632 were chosen as parents because they differ in their ratios of carotenoid isomers. A632 has relatively high levels of zeaxanthin and W64a has higher levels of β-carotene, β-cryptoxanthin, and lutein (Weber 1987a). The two parents were crossed to create an F_1; the F_1 was self pollinated to produce the F_2; the F_2 plants were then self pollinated to create a population consisting of 200 $F_{2:3}$ families. The 200 $F_{2:3}$ families were testcrossed to AE335, an inbred developed from the Alexander Elite population. AE335 was chosen for its higher oil level (approx. 8.0%) in the kernel. A total of 185 testcross families were successfully created by hand pollinations and used in the testcross evaluations.

Field evaluation

The 200 $F_{2:3}$ families and inbred checks were grown in 2 years (1996 and 1997) at the University of Illinois Crop Sciences Research and Education Center, Urbana, Illinois. The checks were the two parents, A632 and W64a, each planted twice, and six lines—B37, A619, and four experimental RSSC lines—chosen for differences in carotenoid and tocopherol levels. The $F_{2:3}$ families plus checks were planted in an alpha (0,1)-design, with 42 blocks and five families per block,with two replications each year. The rows were 5 m long, with a between-row distance of 0.76 m. Each plot was over-planted and thinned to a plant density of approximately 43,000 plants per hectare. Five to seven $F_{2:3}$ plants per plot were self-pollinated. F_4 seed from the F_3 plants within each F_2 family was harvested and bulked at shelling. An aliquot of approximately 15 ml of seed was ground and analyzed for carotenoid concentration.

A total of 185 hybrids and ten checks, including each parent crossed to the AE335 tester, were grown in 3 years (1998, 1999 and 2000) at the University of Illinois Research and Education Center, Urbana. The testcrosses and checks were planted in a randomized complete block design with two replications per year. The hybrids were planted in two-row plots, with each plot 5.3 m long and 0.76 m between rows. Each plot was over-planted and then thinned to a final plant density of approximately 57,000 plants per hectare. Plants were allowed to open-pollinate. Plots were harvested with a research plot combine. An approximately 100-g sample of grain was kept from each plot for measurement of kernel composition. The 1998 data were not used because of a poor growing season resulting in multiple missing plots, thus only data from 1999 and 2000 were analyzed.

Carotenoid extraction and quantification

The vitamin extraction procedure used (Kurilich and Juvik 1999) is a modification of a procedure initially described by Weber (1987a). Carotenoid concentrations in the per se and testcross progenies were determined using a high-performance liquid chromatographer (HPLC). The HPLC procedure is a modification of methods described previously (Weber 1987a; Hart and Scott 1995; Kurilich and Juvik 1999) and is comprehensively outlined in a companion paper (Wong et al. 2003). Quantification of compounds was accomplished by standard regression with external standards. The standard for β-carotene was purchased from Sigma (St. Louis, Mo.), while those for β-cryptoxanthin, lutein, and zeaxanthin were purchased from Extrasynthase (France). The standards were brought to the correct concentration using a method from National Institute of Standards and Technology (National Institute of Standards and Technology 1994). Four dilutions were made allowing for the calculation of the standard regression and quantification of the individual carotenoids. Most HPLC analyses were done within 24 h of extraction to avoid sample degradation. If

the samples could not be assayed within 24 h, they were stored at 4°C in the dark until loaded in the HPLC system.

Two different HPLC systems with a different column and detector were used for quantifying the carotenoids in the per se (System I) and testcross (System II) progenies because of the purchase of a new Waters HPLC system (Milford). The per se progenies were assayed with System I, which used two columns connected in series—a Vydac 4.6×150-mm reverse phase column and a Waters Nova-Pak C-18 3.9×150 mm column. A multi-wavelength detector set at 450 nm with AUFS=0.8 was used. The flow rate was 1.8 ml min^{-1}, with each run taking approximately 18–22 min. Fifty-microliter aliquots of samples were loaded into WISP 1-ml brown injection vials with 200-μl limited volume inserts with self-centering springs (Alltech and Associates).

Testcross progenies were quantified with System II using a YMC carotenoid C-30 column (5 μm, 4.6×100 mm) that has been shown to separate carotenoids better than other columns (Darnoko et al. 2000; Sander et al. 2000). A photo-diode array detector was used for detecting carotenoids in the testcross progenies. Samples were loaded into amber glass containers with 50-μl limited volume inserts. Carotenoids were detected at 450 nm at a flow rate of 1.8 ml min^{-1}, with each run taking 15–18 min. To control fluctuations in retention time due to temperature changes, we set the column temperature at 30°C; to control degradation of the samples by heat, we set the sample temperature at 4°C.

DNA isolation and genotypic analysis

Balanced bulks were created from individual ears of F_4 seed from the four replicates grown and harvested in 1996 and 1997. Approximately 25–28 ears of seed, representing 25–28 F_3 kernels of seed from each F_2 family were used to create the bulk samples. From these balanced bulks of the 200 $F_{2:3}$ families, 30 random kernels were planted in the greenhouse for tissue sampling. Tissue was cut from young plants, ground under liquid nitrogen, and stored at −80°C until DNA isolation could be done. DNA was isolated following the procedure described by Mikkilineni (1997). Microsatellites (SSR, simple sequence repeats) were used for molecular marker mapping (Senior et al. 1996). The mapping population parents, W64a and A632, were screened with 531 markers from the MaizeDB Public SSR list (Maize Database 2002). Of the markers screened, 163 were polymorphic for the cross and subsequently assayed on the 200 $F_{2:3}$ family mapping population; 123 of these markers were reliably scored for the progenies. An aliquot of DNA from 200 $F_{2:3}$ families was placed into 96-well plates and diluted 50 times with water. All reactions were run using a PTC-100 with 96-V-Bottom Well thermocycler (MJ Research, Waltham, Mass.). The microsatellite procedure is a modification of the procedure outlined by Senior et al. (1996). Following amplification, samples were stored at 4°C until the reaction could be evaluated on gels. The PCR reaction products were separated by gel electrophoresis. Bands were scored as 0.5 for homozygous A632, −0.5 for homozygous W64a, and 0.0 for heterozygotes.

Statistical analysis

Phenotypic analyses were performed using the SAS statistical software package (SAS Institute 1996). The four carotenoid compounds, β-carotene, β-cryptoxanthin, lutein, and zeaxanthin, were analyzed as individual compounds. The summation of values for the individual carotenoids, labeled total carotenoids, was also analyzed. Means, range of means, and variances were calculated for individual and combined years on the complete unadjusted data set using the mean procedure (PROC MEANS) in SAS. Significance of effects for the Alpha design (Federer and Wolfinger 1998) for the per se progenies and the RCB design of the testcrosses were tested using the generalized linear model procedure (PROC GLM) of SAS. Effects for year, location, replication, block and families were to be considered random for both per se and testcross progenies. Heritabilities were calculated using the covariance estimates from PROC MIXED (Littell et al. 1996b). For the per se progenies, PROC GLM was used for data analysis. Effects of year, replications (year), block (year and replications), family, and family by year were included in the model. For the testcross progeny, the model included effects of year, replications (year), family, and family by year. Best linear unbiased predictor (BLUP) estimates of family values for all traits were used in the marker analysis (Littell et al. 1996a). Initial QTL detection was done by single factor analysis of variance using the PROC GLM statement.

JOINMAP version 3 (Ooijen and Voorrips 2001) was used to construct a linkage map for the molecular markers used. Prior to mapping, JOINMAP data analysis tools were used to evaluate the quality of the molecular marker data. Data were screened for missing data points, segregation distortion, and similarity between markers or individuals, and those markers with a high level of segregation distortion or missing values were removed. The initial linkage grouping of markers was done at a LOD threshold of 5.5. Groups were joined together based on previous mapping data for these markers (Maize Database 2002). The mapping function of Haldane was used in the creation of maps in each group. Initial map positions within each group were checked against the maize composite maps (Maize Database 2002) for correspondence, and markers that did not correspond were discarded. The final map included 111 out of the 123 markers assayed on the progenies, with a total genome length of 1,645.9 centimorgans (cM) and an average distance between markers of 16.3 cM (Fig. 3).

PLABQTL (Utz and Melchinger 1996) was used for composite interval mapping (CIM). The standard F_2 and testcross options of PLABQTL were used for the per se and testcrosses. The "Permut 1000" statement was performed within PLABQTL to calculate critical LOD values for detection of QTL for each trait. For individual and total carotenoids, an experiment-wise error rate of $P \leq 0.25$ was calculated as having a critical LOD ranging from 2.79 to 2.89 and 2.93 to 3.00 for the per se and testcross progenies, respectively. Putative QTL with a LOD value greater than 3.0 for the per se and testcross population will be presented. Cofactors used for calculating the CIM were selected by PLABQTL by stepwise regression using the COV SELECT option. LOD curves were created by scanning every 2 cM of all possible linkage groups. QTL in adjacent intervals were considered to be the same QTL, due to limitations in the PLABQTL program (F. Utz, personal communication). Digenic epistatic interactions were tested in the initial models using the model AA commands in PLABQTL. For the per se population, dominant effects were also included using the MODEL D command. The phenotypic variation accounted for by an individual QTL (R^2) was calculated as the square of the partial correlation coefficient from the final multiple regression model. The proportion of the genetic variance accounted for by the marker model was calculated as R^2 divided by heritability (Dudley 1993).

Results and discussion

Descriptive statistics

Means and range of values for the carotenoids for the per se and testcross progenies are presented in Table 1. For the per se Progenies the combined year means for β-carotene, β-cryptoxanthin, zeaxanthin, and lutein were 0.69, 2.64, 8.33, and 4.35 μg g^{-1}, respectively. β-carotene and β-cryptoxanthin had higher values in 1996, with means of 1.05 μg g^{-1} and 3.94 μg g^{-1}, versus 0.33 μg g^{-1} and 1.30 μg g^{-1} in 1997, respectively. For zeaxanthin, the opposite trend was apparent: 1997 had a mean of 10.64 μg g^{-1} versus 6.09 μg g^{-1} in 1996. Differences between years with respect to lutein concentration were small. The carotene compound, α-carotene, was measurable in the per se progenies but was detected at a very low

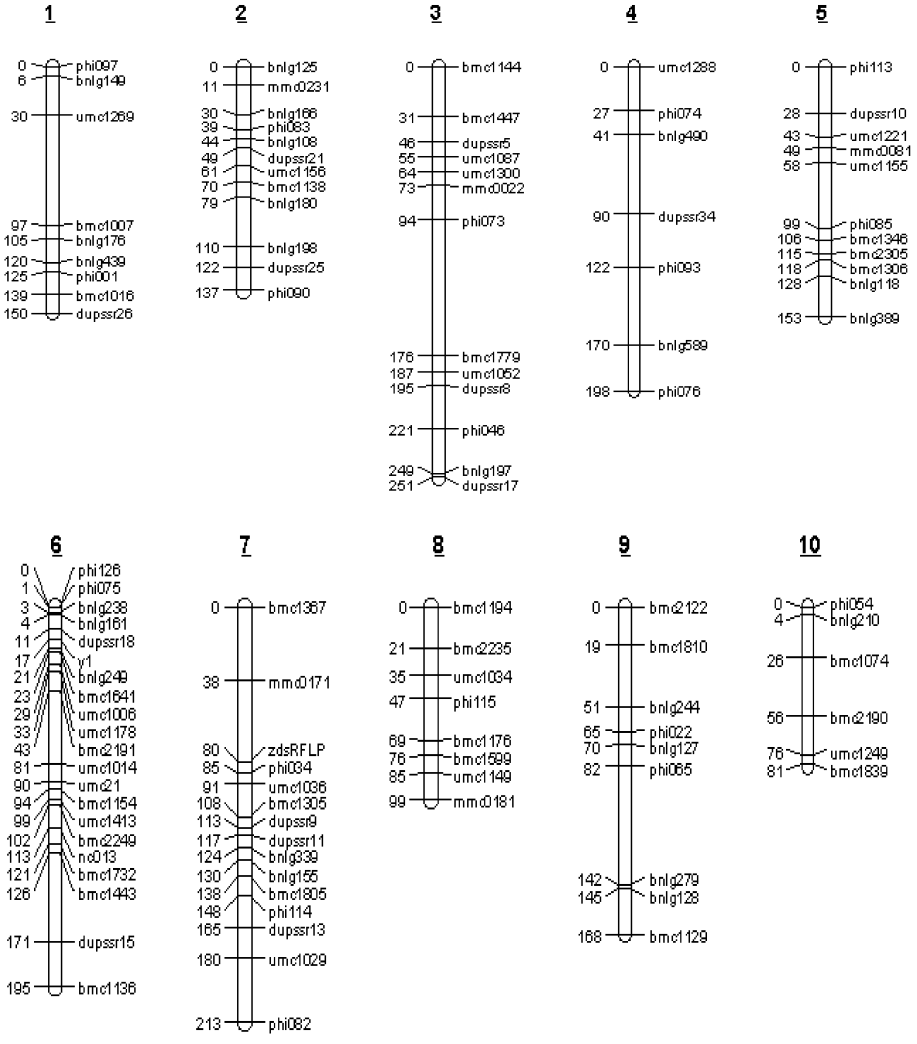

Fig. 3 Genetic linkage map for (W64a × A632) $F_{2:3}$ mapping population

concentration with a mean of 0.16 μg g^{-1} (data not shown).

In the testcross progenies, β-carotene, β-cryptoxanthin, zeaxanthin, and lutein had combined year means of 0.95, 1.92, 7.54, and 10.87 μg g^{-1}, respectively (Table 1). For individual years, the values of most of the carotenoids were similar, except for lutein, which had a higher mean in 2000 of 12.02 μg g^{-1} versus 9.69 μg g^{-1} in 1999. Of the carotenoids tested, in both the per se and testcross progenies, β-carotene was present at the lowest concentration. The compound with the highest concentration differed between the two sets of materials: zeaxanthin was highest in the per se population, whereas lutein was highest in the testcrosses.

Total carotenoids in the per se and testcross progenies (Table 1) had means of 15.73μg g^{-1} and 21.29 μg g^{-1}, respectively. The levels of total carotenoids in the per se and testcross progenies did not differ much between years, even though the concentration of individual carotenoid compounds did fluctuate. In the per se progenies, β-carotene and β-cryptoxanthin were detectable at higher levels in 1996 and zeaxanthin was present at a higher level in 1997; for the testcross progenies, lutein was present at a higher level in 2000 than in 1999. The stability of total carotenoids over years may be related to the relationship between the individual carotenoid compounds. As the level of one compound increased, the levels of other compounds in the pathway decreased, thereby maintaining a relatively stable level of total carotenoids. This is consistent with the relationship of the individual carotenoid compounds produced within the carotenoid biosynthetic pathway (Fig. 2).

The analysis of variance for the per se and testcross progenies showed significant differences for family means for all traits except β-carotene in the testcross progenies (Data not shown). Therefore, β-carotene was not analyzed for QTL in the testcross progenies.

Heritabilities were estimated for the concentrations of all carotenoids studied in both the per se and testcross progenies. Heritabilities in the per se progenies were 48%, 61%, 72%, 83%, and 87% for β-carotene, β-cryptoxanthin, zeaxanthin, lutein, and total carotenoids, respectively. The heritabilities in the testcross progenies were 0%, 43%, 43%, 71%, and 49% for β-carotene, β-cryptoxanthin, zeaxanthin, lutein, and total carotenoids, respectively. No phenotypic variation was estimated for β-carotene,

Table 1 Year means, standard errors of the mean, and range of means of carotenoids for 200 $F_{2:3}$ lines derived from the cross W64a × A632 evaluated in 1996, 1997, and combined across years, and for 185 testcross progenies with AE335 evaluated in 1999, 2000, and combined across years. Two-year means for parents A632 and W64A, and their testcross progenies with AE335 are also provided

Variable	Per se				Testcross			
	Year	Mean ($\mu g\ g^{-1}$)	Standard error	Minimum–maximum ($\mu g\ g^{-1}$)	Year	Mean ($\mu g\ g^{-1}$)	Standard error	Minimum–maximum ($\mu g\ g^{-1}$)
β-Carotene	1996	1.05	0.08	0.08–7.31	1999	0.94	0.02	0.11–1.95
	1997	0.33	0.01	0.08–2.11	2000	0.96	0.03	0.03–1.62
	Combined	0.69	0.04	0.12–3.97	Combined	0.95	0.02	0.40–1.54
	W64a	0.32	0.06	0.10–0.58	W64a x AE335	0.63	0.17	0.14–1.18
	A632	0.26	0.06	0.07–0.44	A632 x AE335	1.01	0.28	0.01–1.70
β-Cryptoxanthin	1996	3.94	0.24	0.48–16.85	1999	2.02	0.04	0.09–3.34
	1997	1.30	0.04	0.22–3.39	2000	1.83	0.04	0.72–3.16
	Combined	2.64	0.13	0.37–10.07	Combined	1.92	0.03	0.40–2.96
	W64a	1.41	0.34	0.21–2.26	W64a x AE335	1.60	0.24	0.25–2.29
	A632	0.99	0.08	0.83–1.27	A632 x AE335	2.07	0.42	0.14–2.94
Zeaxanthin	1996	6.09	0.09	2.45–10.92	1999	7.17	0.07	4.37–9.88
	1997	10.64	0.19	1.58–20.95	2000	7.91	0.14	3.34–11.41
	Combined	8.33	0.13	3.81–15.46	Combined	7.54	0.08	4.64–10.12
	W64a	7.94	1.06	2.41–11.88	W64a x AE335	6.94	0.52	4.71–8.36
	A632	8.66	0.90	6.18–12.71	A632 x AE335	9.32	0.32	7.41–10.53
Lutein	1996	4.95	0.16	0.78–18.79	1999	9.69	0.11	4.88–13.87
	1997	3.59	0.17	0.05–15.17	2000	12.02	0.13	5.20–18.85
	Combined	4.35	0.12	0.67–14.27	Combined	10.87	0.10	6.93–16.35
	W64a	11.59	1.41	8.28–17.99	W64a x AE335	12.12	0.84	8.33–15.12
	A632	2.47	1.19	1.09–6.03	A632 x AE335	10.37	0.47	8.27–11.95
Total carotenoids	1996	16.22	0.41	5.60–39.68	1999	19.83	0.182	13.78–25.74
	1997	15.23	0.30	1.90–40.21	2000	22.71	0.212	11.23–30.37
	Combined	15.73	0.26	5.69–33.21	Combined	21.29	0.150	14.69–27.26
	W64a	17.99	2.88	2.72–30.69	W64a x AE335	21.29	1.67	14.32–26.29
	A632	11.56	1.03	8.47–15.37	A632 x AE335	22.77	0.82	19.50–26.63

so the heritability was zero. The lower heritability estimates in the testcross progenies with respect to those of the per se progenies were expected because the phenotypic variation in the testcross progenies was reduced since all per se progenies were crossed onto the single tester, AE335. Therefore, only one half of the genome segregates among testcross progeny as half the genome in all testcross progeny is from the AE335 inbred tester. Furthermore, the testcrosses were grown in yield trial plots and allowed to open-pollinate. Pollen from adjacent random plots likely pollinated some of the grain and may have altered carotenoid levels in a portion of the grain sampled for analysis, possibly contributing to lower heritabilities.

QTL analysis—per se progenies

As no significant epistatic or dominant effects were detected in the per se progenies, only the additive model was used. CIM identified three chromosomal segments accounting for a large proportion of the total phenotypic variation for all of the individual carotenoid compounds studied (Table 2). These three chromosomal segments were located on chromosomes 6, 7, and 8 (Fig. 3). Interval y1SSR–bnlg249 on chromosome 6 was highly significant for all individual carotenoid compounds and total carotenoids, accounting for 11.8%, 7.1%, 16.3%, 15.6%, and 24.5% of the total phenotypic variation for β-carotene, β-cryptoxanthin, zeaxanthin, lutein, and total carotenoids, respectively. PLABQTL estimated the location of the QTL for the individual carotenoids within 3 cM of the y1SSR marker (Fig. 4), with β-carotene, β-cryptoxanthin, zeaxanthin, and total carotenoids at position 18 and lutein at position 20. The y1SSR marker maps to the *y1* locus (Maize Database 2002).

On chromosome 7, interval zdsRFLP–phi034 was significant for β-carotene, β-cryptoxanthin, zeaxanthin, lutein, and total carotenoids and accounted for 16.6%, 25.4%, 19.8%, 13.1%, and 30.0% of the total phenotypic variation, respectively (Table 2, Fig. 4). The QTL for all individual carotenoids were clustered within 4 cM of marker zdsRFLP (Fig. 4). Marker zdsRFLP is the clone of the ζ-carotene desaturase gene (Luo and Wurtzel 1999), which maps to bin 7.02 and is associated with the *vp9* locus (Maize Database 2002).

On chromosome 8, interval bmc1176–bmc1599 was significant for β-carotene and β-cryptoxanthin at position 72 for both compounds and accounted for 12.1% and 21.4% of the total phenotypic variation, respectively (Table 2). Interval bmc1599–umc1149 at position 78 was significant for lutein and accounted for 49.3% of the total

Table 2 Composite interval mapping results estimated for carotenoid compounds from 200 $F_{2:3}$ lines derived from the cross W64a x A632 and 185 testcross progenies with AE335

Chromosome[a]	Interval	ß-Carotene[b]				ß-Cryptoxanthin				Zeaxanthin				Lutein				Total Carotenoids			
		Position[c] (cM)	LOD	Additive[d] (µg g^{-1})	R^{2e} (%)	Position (cM)	LOD	Additive (µg g^{-1})	R^2 (%)	Position (cM)	LOD	Additive (µg g^{-1})	R^2 (%)	Position (cM)	LOD	Additive (µg g^{-1})	R^2 (%)	Position (cM)	LOD	Additive (µg g^{-1})	R^2 (%)
Per se																					
1	umc1269–bmc1007																	86	3.02	1.15	4.1
3	mmc0022–phi073													74	3.72	0.71	1.1				
4	umc1288–phi074													2	4.69	0.57	7.8	6	3.35	1.06	6.7
5	phi113–dupssr10													28	3.12	–0.42	1.6				
6	Y1SSR–bnlg249	18	8.35	0.08	11.8	18	4.88	0.27	7.1	18	8.72	0.63	16.3	20	8.70	0.78	15.6	18	16.70	2.43	24.5
7	zdsRFLP–phi034	82	9.99	0.09	16.6	82	14.64	0.47	25.4	84	9.35	0.62	19.8	80	7.04	0.64	13.1	82	19.85	2.51	30.0
8	bmc1176–bmc1599	72	3.13	–0.06	12.1	72	11.83	–0.44	21.4	70	2.35	–0.31	6.5								
8	bmc1599–umc1149													78	22.59	1.71	49.3				
9	bnlg127–phi065	72	3.97	0.12	1.6																
Testcross																					
6	dupssr18–y1SSR													14	9.26	0.49	13.4				
6	bmc1641–umc1006																	24	2.83	0.23	7.6
7	phi114–dupssr13									148	3.14	–0.14	4.4					150	2.39	–0.24	3.8
8	phi115–bmc1176									54	6.44	–0.18	11.6								
8	bmc1176–bmc1599					74	3.54	–0.06	10.7												
8	bmc1599–umc1149													78	14.16	0.60	24.5				
8	umc1149–mmc0181																	88	2.21	0.26	3.3

[a] Chromosome location based on the Missouri Maize Consensus map
[b] Not evaluated in testcross progenies
[c] Position from the top of the chromosome as calculated by PLABQTL
[d] Additive effect as calculated by PLABQTL
[e] Proportion of phenotypic variation accounted for by each QTL calculated by multiple regression in PLABQTL

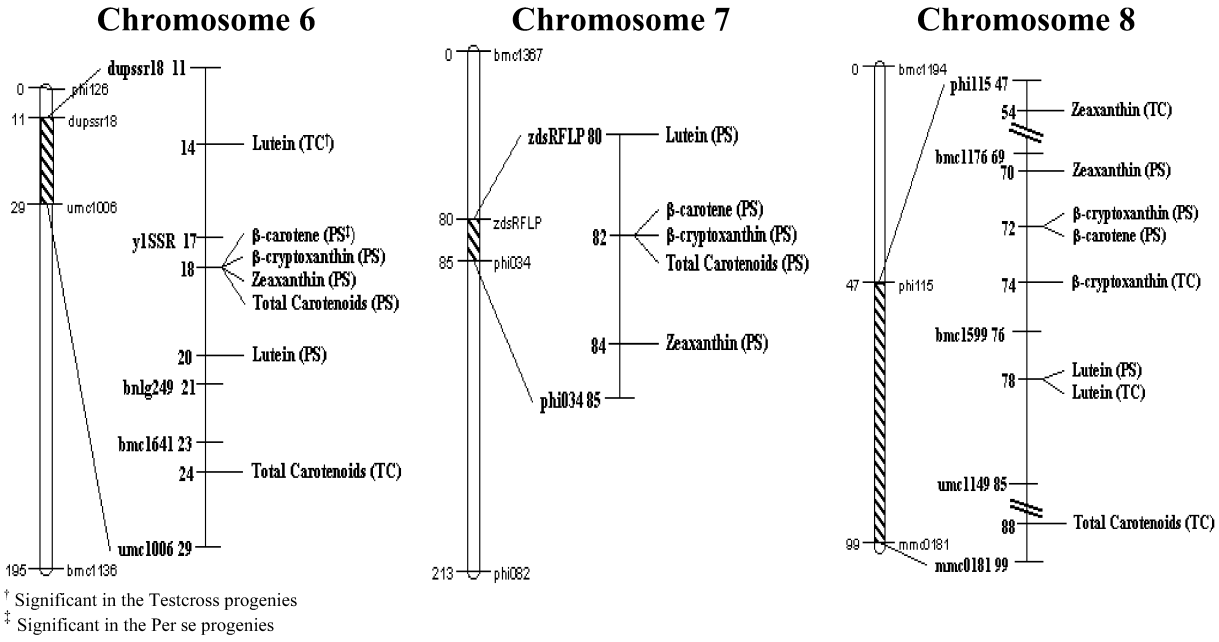

Fig. 4 Putative locations of QTL for carotenoids relative to markers on the final map for chromosomes 6, 7, and 8. QTL positions for 200 (W64a × A632) $F_{2:3}$ per se progenies and the 185 (W64a × A632 $F_{2:3}$) × AE335 testcross progenies shown are relative to their location on the (W64a × A632) $F_{2:3}$ per se map

phenotypic variation. PLABQTL did locate a QTL with a subthreshold LOD value of 2.35 for zeaxanthin at position 70 that accounted for 6.5% of the total phenotypic variation. Thus, the QTL for β-carotene, β-cryptoxanthin, lutein, and zeaxanthin were clustered within 6 cM of marker bmc1599 (Fig. 4).

QTL analysis—testcross progenies

No significant epistatic effects were detected in the testcross progenies, so only the additive model was used. CIM identified intervals accounting for a significant proportion of the total phenotypic variation for individual carotenoid compounds, but no one interval was significant for all of the individual compounds studied. Three intervals on chromosome 8 contained QTL for the individual carotenoids. Interval phi115–bmc1176 at position 54 accounted for 11.6% of the total phenotypic variation for zeaxanthin; interval bmc1176–bmc1599 at position 74 accounted for 10.7% of the total phenotypic variation for β-cryptoxanthin; interval bmc1599–umc1149 at position 78 accounted for 24.5% of the total phenotypic variation for lutein (Table 2). The QTL for β-cryptoxanthin and lutein were in adjacent intervals clustered near marker bmc1599 (Fig. 4).

Three intervals for total carotenoids were identified in the testcross progenies when the lower LOD threshold of 2.0 was used. Interval bmc1641–umc1006 on chromosome 6 accounted for the highest proportion of phenotypic variation of the QTL detected, with 7.6%; phi114–dupssr13 on chromosome 7 accounted for 3.8%; umc1149–mmc0181 on chromosome 8 accounted for 3.3% of the total phenotypic variation for total carotenoids.

Comparison of per se and testcross results

Intervals on chromosome 8 showed QTL in both the per se and testcross progenies for individual carotenoid compounds. Interval bmc1176–bmc1599 had QTL for β-cryptoxanthin for both the per se and testcross progenies at positions 72 and 74, respectively. Interval bmc1599–umc1149 had QTL for lutein, with the QTL at position 78 in both populations. The QTL for zeaxanthin in the two populations were located in two adjacent intervals, phi115–bmc1176 at position 54 in the testcross progenies and bmc1176–bmc1599 at position 70 in the per se progenies. The QTL for zeaxanthin are in adjacent intervals, but the distance between the positions of the QTL is relatively large—16 cM. Notably, the LOD value for zeaxanthin in the per se progenies (2.52) was much lower than that in the testcross progenies (14.8).

The intervals on chromosome 8 with QTL for individual carotenoids are not yet associated with candidate genes. These intervals have QTL for all of the individual compounds in both the per se and testcross progenies and account for a large proportion of the phenotypic variation for β-cryptoxanthin and lutein. β-Cryptoxanthin and lutein do not appear to be very closely related in the carotenoid biosynthetic pathway. β-carotene is the precursor to β-cryptoxanthin and α-carotene is the precursor to lutein (Fig. 2). β-Cryptoxanthin and lutein do have in common the addition of a hydroxyl group to the β-ring of their precursor compounds, β-carotene and α-

carotene, respectively. The QTL on chromosome 8 may influence the rate of the addition of the hydroxyl group to the β-ring, thereby affecting β-cryptoxanthin and lutein levels and, consequently, the levels of the precursor compounds.

The final multiple regression model for the per se progenies identified four QTL for β-carotene, three for β-cryptoxanthin, six for lutein, and three for zeaxanthin. The final models for β-carotene, β-cryptoxanthin, zeaxanthin, and lutein accounted for 28.3%, 36.7%, 25.5%, and 57.1% of the total phenotypic variation, respectively (Table 3). The amount of genotypic variation accounted for was 59.0%, 60.1%, 35.4%, and 67.2% for β-carotene, β-cryptoxanthin, zeaxanthin, and lutein, respectively (Table 3). In the testcross progenies, β-cryptoxanthin had one QTL, and both zeaxanthin and lutein had two QTL. The final models for the testcross progenies for β-cryptoxanthin, zeaxanthin, and lutein accounted for 9.7%, 14.6%, and 30.8% of the total phenotypic variation, respectively (Table 3). These QTL accounted for 22.6%, 34.0%, and 43.4% of the genotypic variation for β-cryptoxanthin, zeaxanthin, and lutein, respectively (Table 3). The testcross progenies had fewer QTL detected than the per se progenies, accounting for much less of the phenotypic variation. The genotypic variation explained by the models for per se and testcross progenies were similar for zeaxanthin, accounting for 35.4% and 34.0%, respectively. For β-cryptoxanthin and lutein, the amount of genotypic variation accounted for in the per se progenies was higher than in the testcross progenies: 60.1% and 67.2% in the per se progenies versus 22.6% and 43.4% in the testcross progenies, respectively.

Two chromosomal regions adjacent to the candidate genes *psy* and *zds* respectively were found to be not significant for QTL analysis of individual carotenoids in the testcross progenies. All individual carotenoid compounds in the per se progenies were significant on chromosome 6 and 7, near y1SSR and *zds*RFLP, accounting for a substantial amount of phenotypic variation for the individual compounds. However, these regions were not significant in the testcross progenies with the exception of a QTL for lutein near y1, which showed a peak at position 14 in comparison to position 20 in the per se progenies.

Chromosomes 6 and 8 had QTL for total carotenoids in both the per se and testcross populations (Fig. 3). On chromosome 6, the QTL for total carotenoids in the per se progenies was located in interval y1SSR–bnlg249; for the testcross progenies, this QTL was in interval bmc1644–umc1006. The peak positions of the QTL on chromosome 6 in the two progeny sets were not close, at position 18 for the per se population and position 24 for the testcross.

The final model for total carotenoids identified three and four QTL in the per se and testcross progenies, respectively, accounting for 43.3% and 10.1% of the total phenotypic variation (Table 3). The model for per se progenies accounted for a larger amount of genotypic variation than that for the testcross progenies, with 49.8% in the per se and 20.6% in the testcrosses (Table 3). It is noteworthy that the detection of QTL for total carotenoids in the testcross progenies required a lowering of the LOD threshold to 2.0 versus 3.0 in the per se progenies. The LOD values ranged from 2.21 to 2.83 in the testcross progenies versus 3.02 to 20.07 in the per se progenies.

Table 3 Number of QTL (*n*), proportion of phenotypic variation explained (R^2), and proportion of genotypic variation explained (p) for the carotenoids in the 200 (W64a x A632) $F_{2:3}$ per se progenies and the 185 (W64a x A632 $F_{2:3}$) x AE335 testcross progenies

	Per se			Testcross		
	n	R^2 (%)	p (%)	*n*	R^2 (%)	p (%)
β-Carotene[a]	4	28.3	59.0	–	–	–
β-Cryptoxanthin	3	36.7	60.1	1	9.7	22.6
Zeaxanthin	2	25.5	35.4	2	14.6	34.0
Lutein	6	57.1	67.2	2	30.8	43.4
Total carotenoids	4	43.3	49.8	3	10.1	20.6

[a] Not evaluated in testcross progenies

AE335 had carotenoid concentrations of 0.26, 0.58, 4.92, and 2.17 μg g^{-1} for β-carotene, β-cryptoxanthin, zeaxanthin, and lutein, respectively (data not shown). The concentrations of each of the four carotenoids in AE335 were lower than those detected in both of the inbred parents and the mean concentrations of the per se progenies. Thus, the tester did not have relatively higher concentrations of the carotenoid compounds, which may have masked the detection of the QTL detected in the per se progenies. β-carotene and lutein had higher means in the testcross progenies than in the per se progenies, whereas β-cryptoxanthin and zeaxanthin had lower means in the testcross progenies than in the per se progenies. These differences may have affected the detection of QTL in the per se progenies versus the testcross progenies. However, the relative effect of the allele in the tester with respect to the two alleles segregating in the per se progenies at an individual locus will have a large influence on whether a QTL that is detected in the per se progenies is also detected in the testcrosses.

Interpretation and perspective

One of the noteworthy aspects of this study is the interrelationship among different carotenoid compounds due to their common biosynthetic pathway, with some compounds being precursors to other compounds. Therefore, QTL with effects on the levels of one carotenoid may also affect the levels of other carotenoids. Some of the major QTL identified in this study affect more than one carotenoid. Most of the major QTL detected for the individual carotenoids are in the same or adjacent interval, suggesting a single QTL might be affecting more than one compound. In some cases, PLABQTL located the peaks for individual carotenoids at slightly different locations within the same interval. The discrepancy between the locations of peaks of related compounds is likely due to the differences in experimental error

associated with the collection of the phenotypic data of the different carotenoids. It also possible that there are linked genes that influence the locations.

PLABQTL tends to locate peaks of QTL somewhere near the center of the interval because of the method used to calculate position. The center of the interval allows more error to be explained by possible recombination (F. Utz, personal communication). The tendency to place the peak in the middle of an interval could be a source of error when comparing the peak positions of closely related traits such as individual carotenoids. It is possible that the larger the distance between the flanking markers, the greater the chance of having the peak position of individual carotenoids differ by a few to several centi-Morgans. The possibility also exists that in these intervals there may be different genes and/or modifier gene(s) that influence the expression of different carotenoids and, thus, may influence the peak map position of QTL for the different carotenoids. However, it is very unlikely that there are different genes modifying the accumulation of different carotenoids in all of the intervals associated with more than one carotenoid in this study.

The genes *y1*, associated with phytoene synthase, and *vp9*, associated with ζ-carotene desaturase, were linked with the observed variation in levels of carotenoids. Both of these genes have also been associated with quantitative variation for carotenoids in the Solanaceae (Thorup et al. 2000). Another gene in the carotenoid biosynthetic pathway, *vp5*, which is associated with phytoene desaturase (*pds*), was not associated with QTL for carotenoids in this study. This observation is similar to that made in the Solanaceae study in which the *pds* clone was not associated with quantitative variation for carotenoids.

An important question we were unable to answer in this study is whether the variation we are accounting for is due to the candidate gene locus or if it is associated with another gene linked to the candidate gene. Mapping populations, such as the one in this study, or populations with very large numbers of progeny do not provide enough precision to resolve this question. Thorup et al. (2000) hypothesize that because carotenoids are so important to plant health, most of the variation associated with these genes is likely due to *cis*-acting regulatory sequences, not structural mutations in the coding regions. It is possible that the quantitative variation we and Thorup et al. (2000) detect is due to nucleotide variation in the promoter, intron, or the 3′ regions of the gene, and not to the coding region of the candidate gene. There could be sequences considerably 5′ to the promoter region that influence the level of transcription of the gene. This type of variation could influence the abundance of mRNAs and translated enzyme. Alternatively, there could be minor nucleotide variation in the coding region that influences the efficiency of the enzyme. We plan to sequence the candidate genes *psy* and *zds* and flanking 5′ and 3′ sequences in the inbred parents of our mapping population to search for allelic differences. We also plan to sequence *psy* and *zds* in a set of genetically diverse maize inbreds which are part of the ongoing NSF Plant Genome Project, Evolutionary Genomics. (http://plantgenome.sdsc.edu/AwardeesMeeting/poster_Doebley.pdf) and perform associative genetic analyses (Remington et al. 2001). We will search for nucleotide variation in or near these candidate genes that appears to be associated with levels of carotenoids in a manner similar to what has been shown for variation for plant height and allelic variation in the *dwarf8* gene (Thornsberry et al. 2001). If associative genetic analyses suggest functional differences associated with allelic variation in *psy* and *zds*, then efforts could be made to survey the DNA sequence of *psy* and *zds* in diverse germplasm sources to potentially identify alleles that have larger, more favorable effects. Subsequently, efforts could be considered to directly select the more favorable allele in marker-assisted selection programs. Analysis of levels of transcription of alleles of *psy* and *zds* will also be performed. These two approaches may provide useful information for determining the validity and potential basis of functional allelic variation of the candidate genes.

For grain crops such as maize and rice, there is interest in increasing the levels of β-carotene because it has two pro-vitamin A structures. However, β-cryptoxanthin has a single pro-vitamin A structure and is generally present at higher levels in maize grain than β-carotene. Thus, breeding programs should consider increasing the levels of both compounds. Notably, we detected major QTL significant for both β-carotene and β-cryptoxanthin on chromosomes 6 and 7. Thus, these QTL could be selected to result in increased levels of pro-vitamin A structures. This type of information may be particularly relevant to maize genetic improvement efforts in parts of the world such as sub-Saharan Africa, where maize provides a large portion of the calories consumed. There are widespread vitamin A deficiencies in human populations in sub-Saharan Africa that result in widespread disease. Increased levels of pro-vitamin A in maize grain should help address this problem.

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