RESEARCH

Quantitative Trait Loci Analysis of Endosperm Color and Carotenoid Content in Sorghum Grain

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ABSTRACT

Vitamin A deficiency affects approximately 250 million people in semiarid regions of Africa and Asia, where sorghum (*Sorghum bicolor* Moench) is a major staple crop. Yellow endosperm sorghums contain carotenoids, some of which can be transformed by humans into vitamin A. Our objective was to study the genetic basis of variation in carotenoid levels in sorghum endosperm by mapping quantitative trait loci (QTL) associated with carotenoid content and endosperm color, as a putative predictor of carotenoid concentration. A recombinant inbred line population developed from a yellow ('KS115') by a white endosperm ('Macia') parental cross was evaluated in two locations in 2005. A genetic map was generated using 112 molecular markers including nine carotenoid candidate genes. Lutein, zeaxanthin, and β-carotene were the major carotenoids identified. Several QTL were detected for each compound as well as for color and total carotenoids. Color was significantly correlated with the levels of all compounds, and color QTL co-localized with carotenoid QTL. For β-carotene (provitamin A), five QTL were localized on chromosomes 1, 2, and 10. One of them, on chromosome 2, was stable across both environments, had positive additive effects (1.179 and 1.379), explained large proportions of the phenotypic variance (11.6% and 15.15%), and was associated with a new phytoene synthase gene (*Psy3*). This first report of QTL for carotenoid content in sorghum grain provides a starting point for breeding high-provitamin A sorghums.

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Abbreviations: CCD, carotenoid cleavage dioxygenase; CIM, composite interval mapping; CRTISO, carotenoid isomerase; GGDP, geranylgeranyl diphosphate; HPLC, high performance liquid chromatography; INDELS, insertion or deletion; LCYB, lycopene β-cyclase; LCYE, lycopene ε-cyclase; LOD, logarithm of the odds; LR, likelihood ratio; MAS, marker-assisted selection; PDS, phytoene desaturase; PSY, phytoene synthase; RFLP, restriction fragment length polymorphism; RIL, recombinant inbred line; SMA, single marker analysis; SSR, simple sequence repeat; QTL, quantitative trait loci; ZDS, ζ-carotene desaturase; ZISO, 15-*cis*-ζ-carotenoid isomerase.

Carotenoids are a group of lipophilic pigments with diverse biological functions. In plants, they are part of light harvesting complexes, involved in photo-oxidative protection, serve as precursors of the hormone abscisic acid and provide color in flowers and fruits to attract pollinators helping to disperse pollen and seeds (Hirschberg, 2001; DellaPenna and Pogson, 2006). In humans, carotenoids have been associated with a reduced risk of cancer, decreased cardiovascular diseases, reduced risk of cataract and age-related macular degeneration, and increased levels of

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iron absorption (Garcia-Casal, 2006; Van den Berg et al., 2000; Mares-Perlman et al., 2002). The best known activity of carotenoids, however, is as precursors of vitamin A (retinol). β-carotene, α-carotene, and β-cryptoxanthin are considered provitamin A compounds with β-carotene having the highest activity, since one molecule can potentially be transformed into two molecules of retinol.

Vitamin A deficiency, affecting approximately 250 million poor people in the semiarid region of Africa and Asia, is the primary cause of blindness, increased mortality in children and pregnant women, and susceptibility to malaria and diarrheal disease (ACC/SCN, 2000). Sorghum (*Sorghum bicolor* Moench) is a critical food cereal crop in those extremely drought-prone and poor regions, where other crops such as maize (*Zea mays* L.) cannot be grown (FAO/GIEWS, 1998; FAO, 1995). In Africa, direct per capita food use of sorghum reaches levels as high as 100 kg a year and 74% of the sorghum produced is consumed by the farmer and his family (FAO, 1995). Therefore, increasing the carotenoid content of sorghum might have a great impact on the health of poor people in the world.

Sorghum grains exhibit a wide variety of colors, ranging from red or purple to white. This color is determined by the allelic constitution of genes affecting pericarp color, mesocarp thickness, pigmented testa, and endosperm color. Endosperm color in sorghum is either yellow or white with a range of variation between the two. Early studies showed that yellow endosperm sorghums had β-carotene contents ranging from 0.22 to 3.23 μg g^{-1} and total carotenoid levels as high as 9 μg g^{-1} (Blessin et al., 1958; Worzella et al., 1965). A recent study evaluating eight sorghum cultivars showed that total carotenoids in grains increased between 10 and 30 d after blooming, but significantly decreased from 30 to 50 d. In fully-matured sorghum kernels, total carotenoid content was 2.62 to 15.02 μg per 1000 kernels, a range significantly lower than average values in maize. Zeaxanthin was the most abundant type of carotenoid and β-carotene concentration was 0.15 to 3.83 μg per 1000 kernels (Kean et al., 2007).

Inheritance studies of endosperm color in sorghum proposed that the trait was oligogenic (Worzella et al., 1965, Gorbet and Weibel, 1972). Although there is limited genetic information about carotenoid biosynthesis in sorghum, the pathway has been thoroughly studied in other species, such as maize, tomato, and *Arabidopsis* (Hirschberg, 2001; Thorup et al., 2000; DellaPenna and Pogson, 2006). Genes encoding major structural enzymes in the pathway have been isolated, mapped, and characterized (Fig. 1). Phytoene synthase (PSY) is the enzyme responsible for the first committed step of the pathway in which two molecules of geranylgeranyl diphosphate (GGDP) are condensed to form phytoene. PSY catalyzes a rate-limiting step and has been found associated with major QTL in several species (Thorup et al., 2000; Wong et al., 2004; Gallagher et al., 2004; Pozniak et al., 2007; Palaisa et al., 2003). In maize, the phytoene synthase family includes PSY1, mainly expressed in leaves, embryo, and in the endosperm, if it is yellow (Gallagher et al., 2004); PSY2, expressed in all tissues and endosperm irrespective of the color (Gallagher et al., 2004); and a recently identified PSY3, highly expressed in embryo and roots particularly in response to abiotic stress (Li et al., 2007b). Phytoene undergoes two sequential desaturation steps catalyzed by phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) to produce lycopene. In maize, ZDS was associated with QTL for lutein, zeaxanthin, β-carotene, β-cryptoxanthin, and total carotenoids (Wong et al., 2004). Isomerization, required to obtain all-trans-lycopene, is catalyzed by two enzymes (Z-ISO and CRTISO) that have been identified in tomato, *Arabidopsis,* and maize (Isaacson et al., 2002; Li et al., 2007a; Park et al., 2002). Subsequently, lycopene β and ε-cyclase (LCYB and LCYE) catalyze the formation of terminal rings and thus control the major branch point in the pathway. The formation of both β- and ε-rings leads to the synthesis of α-carotene and its derivatives. In the other branch of the pathway, two β-rings are formed by LCYB, producing β-carotene. Genes encoding LCYB and LCYE are single copy in most species, except in tomato (DellaPenna and Pogson, 2006). The next step in the pathway leads to the formation of xanthophylls. Lutein and zeaxanthin are synthesized by β- or ε-hydroxylase with the addition of hydroxyl groups. In *Arabidopsis*, three β-hydroxylases and one ε-hydroxylase have been identified (Tian et al., 2004; Kim and DellaPenna, 2006; Tian and DellaPenna, 2004). A group of cleavage enzymes process different carotenoids to produce compounds such as abscisic acid, signaling

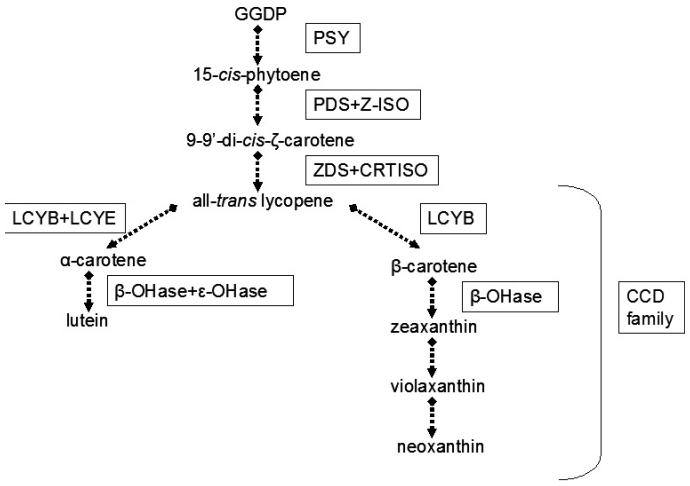

Figure 1. Summary of carotenoid biosynthetic and degradation pathway. PSY, phytoene synthase; PDS, phytoene desaturase; ZISO, 15-*cis*-ζ-carotenoid isomerase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; LCYB, lycopene β-cyclase; LCYE, lycopene ε-cyclase; β-*OHase,* β-ring hydroxylase; ε-OHase, ε-ring hydroxylase; CCD, carotenoid cleavage dioxygenase.

molecules, and volatiles. The first cleavage enzyme was identified in maize from the mutant *vp14*. Nine CCD (carotenoid cleavage dioxygenases) members of this family have been identified in *Arabidopsis* based on homology with VP14 (Tan et al., 2003). CCD1 is a particularly interesting enzyme since its mutants in *Arabidopsis* accumulate high levels of carotenoids in seeds (Auldridge et al., 2006; Booker et al., 2004).

Understanding the genetic mechanisms involved in the synthesis and regulation of carotenoids in sorghum endosperms is a necessary step to breed high-carotenoid sorghums. Our objective was to scan the sorghum genome for quantitative trait loci (QTL) responsible for variation in carotenoid content. To accomplish this, we measured the level of the three major carotenoids in endosperm samples from a recombinant inbred line (RIL) population developed from a cross between 'KS115' (yellow endosperm) and 'Macia' (white endosperm), which exhibit an eight-fold difference in endosperm carotenoid content. The linkage map developed for this research project included markers of some of the major genes in the biosynthetic pathway mentioned above to test the hypothesis that they might be responsible for QTL with major effects, as has been shown in maize (Wong et al., 2004), a close relative of sorghum.

Carotenoid accumulation and biosynthetic gene activity are clearly associated with yellow, orange, or red color in some tissues and species (Shewmaker et al., 1999; Paine et al., 2005; Thorup et al., 2000; Li et al., 2001; Lu et al., 2006). Therefore, we also investigated in the same population the value of sorghum endosperm color as a predictor of carotenoid content. Endosperm color, if highly correlated with carotenoid content and associated with the same major QTL, could be used in a breeding program as a simpler and less expensive phenotypic trait to select.

MATERIALS AND METHODS

Plant Materials and Experimental Design

An RIL population of 351 lines was developed from a cross between KS115 and Macia at Kansas State University and advanced to F_6 generation. KS115 is a yellow endosperm line with extremely large seeds, purple plant color and awns, developed using genetic material originally collected from Sudan (Tuinstra et al., 2001). Macia is a white endosperm line with tan plant color and no awns. This variety is broadly used in South and eastern Africa, and was originally collected from Zimbabwe. The RIL population was planted in College Station, TX and Manhattan, KS during the summer of 2005 in single rows using a randomized complete block design with two replications per location. Three panicles per row were selfed and harvested soon after physiological maturity (determined by black-layer formation). Panicles were dried at room temperature, threshed and decorticated using a tangential abrasive dehulling device (T.A.D.D., Venables Machine Works Ltd., Saskatoon, SK, Canada). Decortication of the grain had a dual purpose, (i) to remove the pericarp so that endosperm color could be measured accurately; and (ii) to remove the embryo as much as possible. Even though removal of the embryo was not 100% effective in all samples, decortication was the most efficient method for such a large number of grains and samples.

Endosperm color was measured on decorticated grain with a Konica Minolta colorimeter CR-300 (Konica Minolta, Ramsey, NJ), which summarizes the color spectrum in three values: a, b, and L. The b value was used in subsequent analysis since it represents the variation in yellow intensity.

Sorghum endosperm was ground using a Cyclone Mill with a 1 mm screen (UDY Corporation, Fort Collins, CO) and maintained at 4°C in the dark until extraction.

Selective phenotyping was performed for carotenoid concentration because of the high cost and time required to run high performance liquid chromatography (HPLC) for a large population with multiple replications. Therefore a subset of 126 RILs was selected for carotenoid analysis based on endosperm color. This subsample of 126 RILs represented all variation found in endosperm color (low, intermediate, and high "b values") with the expectation that they would also capture the variation in carotenoid concentration, since these two traits are correlated in other crops.

Carotenoid Analysis

Extraction of carotenoids was performed on 150 mg of ground endosperm according to Kurilich and Juvik (1999). Combined hexane fractions were dried under a stream of nitrogen and stored at −80°C or immediately resuspended in ethylacetate for HPLC analysis. Carotenoid identification and quantification were done using a reverse-phase chromatography system with a Waters Spherisorb ODS2 C18 analytical column (Waters Corporation, Milford, MA). The elution profile was a 45-min gradient of ethyl acetate in acetonitrile-water-triethylamine (9:1:0.04) at a flow rate of 0.5 mL/minute. Carotenoids were identified by comparison of absorption spectra and retention time with commercial standards and maize samples with known carotenoid profiles. Quantification was done using a regression curve generated with commercial β-carotene and lutein standards (Sigma-Aldrich). Total carotenoids were considered in this analysis as the sum of lutein, zeaxanthin, and β-carotene, since other carotenoids are present at negligible amounts.

Molecular Markers and Genetic Map

DNA of each RIL was extracted from bulked leaf tissue of five plants in the F_6 generation according to Doyle and Doyle (1987). After genotyping and field observations, 39 RILs were discarded; therefore data from 312 RILs was finally used for linkage map construction and endosperm color QTL analysis.

The genetic map was constructed using 112 molecular markers polymorphic between KS115 and Macia, which included 85 microsatellites (SSRs) (Bhattramakki et al., 2000; Casa et al., 2005; Kong et al., 2000), 18 miscellaneous INDELS, and nine INDELS from candidate genes in the carotenoid biosynthetic pathway. The miscellaneous INDELS include 11 PCR-based markers developed from sequences of RFLP probes (Bowers et al., 2003) and seven INDELS from candidate genes in the starch biosynthetic pathway (Hamblin et al., 2007). Sequences of putative sorghum carotenoid candidate genes were assembled from

unannotated genomic and EST sequences by TBLASTN with known protein sequences from other species (see Supplementary Table 1). Portions of these genes were then amplified from KS115 and Macia and sequenced. On the basis of sequence differences, INDELS were developed. Primers and marker information are summarized in the Supplementary Table 2. Candidate genes from the carotenoid pathway are: ε-ring hydroxylase (*Crtre*), β-ring hydroxylase (*Crtrb1*), ζ-carotene desaturase (*Zds*), phytoene desaturase (*Pds*), lycopene ε-cyclase (*Lcye*), two phytoene synthase genes (*Psy1* and *Psy3*), one gene homologous to maize *Ccd1* (*Ccd1a*), and one gene homologous to maize viviparous 14 *(Vp14)*. In this study, TBLASTN results identified two sorghum candidate genes with homology to maize PSY1 protein (AAR08445), so we identified them as *Psy1* and *Psy3*, and tested both of them (Supplementary Table 1). Sorghum *Psy2* gene has been identified by sequence similarity with maize *Psy2* but was not mapped in this study, since in other species, it has not been associated with carotenoid accumulation in the grain (Gallagher et al., 2004). PCR-amplification was performed as previously described (Matsuoka et al., 2002), but for some INDELS PCR amplification was done using a fluorescent universal primer-TAIL system described by Schuelke (2000). Markers were multiplexed in groups of three or four according to expected fragment size and the fluorescent dye used to label each primer (6-FAM, HEX, TET, NED, TAMRA, VIC, or PET; Applied Biosystems Inc., Foster City, CA). Molecular markers were scored using the Applied BioSystems 3730XL DNA Analyzer which determined DNA fragment sizes using fluorescence-based detection and an internal size standard. Fragment sizes were analyzed using GeneMapper Software v3.0 (Applied Biosystems Inc., Foster City, CA).

Some molecular markers had more than two alleles when the RILs were scored. Analysis of multiple sources of seeds from KS115 proved that the inbred line was not completely homozygous and homogenous, and probably contributed more than one allele at the time the population was developed. Therefore, alleles not present in the Macia parent were considered as coming from KS115 for map construction and QTL analysis.

Mapmanager QTX software (Manly et al., 2001) was used to construct the linkage map, which comprised 11 linkage groups with LG10a and LG10b corresponding to sorghum chromosome 10. The map was constructed using the Kosambi mapping function and "make linkage group" command. A core set of markers with no segregation distortion was selected first and used to set the linkage groups. Additional markers with significant segregation distortion were added to the original map using the "distribute" command. The final order of markers was confirmed using the "ripple" command. The linkage criterion was set to $p = 0.0001$ for all commands.

Statistical Analysis

Analysis of variance was performed using JMP 7.0 for Windows (SAS Institute Inc., Cary, NC). Expected Mean Square method was used to fit phenotypic traits to a linear model and to estimate the variance components. The model included the following random effects: locations (L), replications nested within locations (rep(L)), genotypes (G), and genotype by location interaction (G × L).

Heritability for each trait was calculated as $\sigma^2_G/((\sigma^2_E + lr\ \sigma^2_G + r\ \sigma^2_{GL})/lr)$, where l is number of locations, r is the number of repetitions per location, σ^2_G is the genotypic variance, σ^2_E is the residual variance, and σ^2_{GL} is the variance due to genotype × location interaction.

Phenotypic correlation coefficients were calculated using the same software and the "density ellipse" command by which Pearson correlation coefficients were calculated between endosperm color (b values) and each of the carotenoid compounds.

QTL Analysis

Identification of QTL was performed using WinQTL cartographer v.2.5 (Statistical Genetics, NCSU, Raleigh, NC). Linkage between individual markers and putative color or carotenoid QTL was evaluated by single marker analysis (SMA). Analysis was initially performed for individual locations, but because results were coincident, final SMA was performed for each phenotypic value (color, lutein, zeaxanthin, β-carotene, and total

Table 1. Colorimetric estimates of yellow intensity (b values) of decorticated grain from RILs grown at two locations.

Location	N	Reps	Range	Mean	St. Dev.	CV(%)
College Station, TX	312	2	17.7–35.2	24.07	3.069	12.75
Manhattan, KS	312	2	16.7–31.8	22.6	2.772	12.24
Average 2 locations	312	4	17.9–33.5	23.33	2.784	11.9

Table 2. HPLC-measured carotenoid content of selected 126 RILs grown at two locations.

a. Individual compounds

	Lutein				*Zeaxanthin*				β-*carotene*			
Location	Range	Mean	KS115	Macia	Range	Mean	KS115	Macia	Range	Mean	KS115	Macia
	μg/100g				μg/100g				μg/100g			
CS, TX	5.7–58.4	21.86	101.49	8.92	7.1–121.6	37.11	79.66	7.91	0–14.1	4.83	9.01	2.84
MN, KS	7.5–69.7	26.18	65.56	9.17	5.0–87–1	31.85	55.30	9.34	0.4–19	5.30	4.26	1.79
Average	7.9–63	24.02	89.51	9.00	6.0–102.4	34.48	71.54	8.38	0.3–14.8	5.06	7.43	2.49

b. Total carotenoids (sum of lutein, zeaxanthin, and β-carotene)

	Total carotenoids			
Location	Range	Mean	KS115	Macia
	μg/100g			
CS, TX	17.1–177.3	63.81	190.18	19.67
MN, KS	18.36–169.56	63.57	125.13	20.32
Average	19.36–169.56	63.57	168.49	19.89

carotenoid concentration) averaged over both locations and fit to a simple linear regression model. Both likelihood ratio and *F* statistic were calculated and statistical significance established at 5, 1, 0.1, and 0.01% levels.

Endosperm color was additionally analyzed using composite interval mapping (CIM) with averaged "b values" from College Station and Manhattan, since there was a consistent detection of QTL in both environments when analyzed individually. Model 6 of WinQTL cartographer was used with five markers as genetic background control and scanning the genome in 2 cM intervals. Background markers were automatically selected based on results from SrMapQtl. Markers located within 10 cM on either side of the testing site were blocked from the analysis performed using forward regression. The threshold level for significance was established by 1000 permutations at $\alpha = 0.05$.

Individual carotenoid compounds and total carotenoids were evaluated in 126 selected RILs using CIM under the same statistical constraints as described above. Because the proportion of lutein and zeaxanthin differed between locations, phenotypic values were evaluated by location only.

Multiple trait-CIM (Jiang and Zeng, 1995) was subsequently used to analyze endosperm color, individual, and total carotenoid content. In this method, each phenotype (color, lutein, zeaxanthin, β-carotene, and total carotenoids) is evaluated using data from individual locations as correlated traits, resulting in higher power and precision than analyzing each location separately. Multiple trait-CIM was used to confirm the QTL identified by CIM and to test the possibility of detecting new QTL due to the higher power of the method. This method calculates the likelihood ratio (LR) statistic for the alternative hypothesis that the additive effect of the putative QTL on at least one of the traits is not zero. The hypotheses are H0: $b_{k1} = 0$, $b_{k2} = 0$ and H1: $b_{k1} \neq 0$, $b_{k2} \neq 0$, where b_{k1} and b_{k2} are the additive effects of the QTL for trait 1 and 2. As an example, in our study, trait 1 would be "lutein in College Station" and trait 2 would be "lutein in Manhattan". An experiment-wide significance level was established by 1000 permutations for all traits and both analyses (CIM and multiple trait-CIM), at $\alpha = 0.05$.

QTL effects are always expressed relative to the KS115 allele. A positive effect would indicate an increase in the phenotypic value when the KS115 allele was present at that QTL. Likewise, a negative effect indicates that the KS115 allele would reduce the phenotypic value at that particular QTL.

RESULTS

Phenotypic Values and Statistical Analysis

The color of decorticated grain was measured as the "b value," with lower numbers representing the whites and higher values representing the most intense yellow found in this population of 312 lines. Macia averaged 20.88 over the four replications while KS115 averaged 29.41. Transgressive segregation was found, since 18.2% of the RILs had lower b values than the white parent and 2.5% of the RILs had higher b values than KS115. Overall, color values were lower at the Manhattan location (Table 1).

In the subset of 126 RILs selected for carotenoid analysis, average lutein content ranged from 7.96 to 63.0 μg 100 g^{-1} of decorticated grain across locations (Table 2a). Only 0.8% of the RILs had lower lutein levels than Macia and no RIL had higher levels than KS115. However, because this was a subset of RILs selected on the basis of endosperm color, it is possible that some RILs with higher levels than the yellow parent were not included in this subpopulation.

Zeaxanthin was the most abundant carotenoid with average levels ranging from 6.09 to 102.4 μg 100 g^{-1} across locations (Table 2a). Only 0.8% of the RILs had lower zeaxanthin content than the white parent and 5.5% of the RILs had higher content than the yellow parent. While zeaxanthin was the predominant carotenoid in both locations, the relative levels of lutein and zeaxanthin varied between locations. In College Station, lutein represented 34% of the total carotenoids, while in Manhattan, it increased to 41%. Zeaxanthin levels varied accordingly, being 58% in College Station and 50% in Manhattan. This compensating balance between the xanthophylls maintained a stable level of total carotenoids over both locations. Even though KS115 had higher levels of lutein than zeaxanthin, 64% of the RILs in College Station and 57% of the RILs in Manhattan showed the opposite trend, with zeaxanthin levels being higher than lutein.

β-carotene was the least abundant carotenoid, with 18% and 20% of the RILs having β-carotene content lower and higher than their white and yellow parents, respectively (Table 2a).

Total carotenoids were calculated as the sum of lutein, zeaxanthin, and β-carotene, as these were the major pigments quantified in this study. The yellow parent (KS115) had an average total carotenoid content of 168.49 μg 100 g^{-1}; only one RIL had higher levels. The white parent (Macia) averaged 19.89μg 100 g^{-1} of total carotenoids; only one RIL had lower levels (Table 2b).

The level of heterozygozity was 3.16%, similar to what is expected for an F_6 generation. The analysis of variance showed that all effects were significant except replications nested within locations for zeaxanthin, and locations for lutein, β-carotene, and total carotenoids. In all four cases, the variance component due to differences in genotypes explained the highest proportion of the total phenotypic variance (Tables 3a and b). This is reflected in the high trait heritabilities: 90.7% for endosperm color, measured as b value, and 83.8, 89, 90.5, and 91% for lutein, zeaxanthin, β-carotene, and total carotenoids, respectively.

Correlation coefficients were calculated between phenotypic values of each carotenoid compound and endosperm color. Yellow color was positively correlated with concentration of all carotenoids. The highest correlation was between total carotenoids and color (0.71), intermediate in the case of β-carotene (0.59) or zeaxanthin (0.69), and lowest between lutein and endosperm color (0.42). All correlations were statistically significant at $p \leq 0.0001$.

Table 3. a. Estimates of variance components and percentage of the total variance they represent.

Variance component	lutein		zeaxanthin		β-carotene		Total carotenoids		Color (b value)	
	Estimate	% total	Estimate	% total	Estimate	% total	Estimate	% total	Estimate	% total
σ^2_L	3.26	2.11	12.51	2.08	–0.17	–1.59	–5.82	–0.50	0.94	9.82
$\sigma^2_{R(L)}$	12.99	8.41	0.79	0.13	0.58	5.20	8.96	0.78	0.07	0.73
σ^2_G	83.73	54.19	427.95	71.25	8.24	73.39	862.02	75.30	7.12	74.39
σ^2_{GXL}	10.40	6.73	48.19	8.02	0.78	7.01	62.76	5.48	0.45	4.70
σ^2_E	44.10	28.54	111.17	18.51	1.79	15.98	216.81	18.99	1.93	20.16

b. ANOVA F statistics and significance levels for each effect.

Variance component	lutein		zeaxanthin		β-carotene		Total carotenoids		Color (b value)	
	F value	Prob (F)	*F* value	Prob (F)	*F* value	Prob (F)	*F* value	Prob (F)	*F* value	Prob (F)
σ^2_L	1.48	0.344	11.18	0.0273	0.41	0.584	0.005	0.9447	316.17	<0.0001
$\sigma^2_{R(L)}$	37.38	<0.0001	1.88	0.1546	41.20	<0.0001	6.105	0.0026	14.66	<0.0001
σ^2_G	6.11	<0.0001	9.18	<0.0001	10.71	<0.0001	10.98	<0.0001	15.69	<0.0001
σ^2_{GXL}	1.46	0.0059	1.85	<0.0001	1.86	<0.0001	1.57	0.0014	1.54	<0.0001

Genetic Map

A genetic map was constructed based on 112 markers genotyped in the total population of 312 RILs. This map was, in general, consistent with previous sorghum maps (Menz et al., 2002; Bowers et al., 2003; Schloss et al., 2002). Markers mapped to eleven linkage groups corresponding to one linkage group each for chromosomes 1 to 9, and two linkage groups for chromosome 10 (Fig. 2). LG 10a and 10b could not be linked since no polymorphic markers were identified in a region of 35 cM according to previous maps (Menz et al., 2002). The total map length was 1364.6 cM with an average interval between markers of 13.51 cM. Some of the markers had not been previously mapped in sorghum. These include the candidate genes from the carotenoid biosynthetic pathway (*Ccd1a, Vp14, Crtrb1, Crtre, Lcye, Zds, Pds, Psy1,* and *Psy3*) as well as several genes from the starch biosynthetic pathway (*Pgm, Agpls, Dbei, SSIII, Gpt, SSI,* and *SSII*). Carotenoid candidate genes are shown in bold in Fig. 2. The preliminary assembly of the sorghum genome supports most of these positions.

QTL Analysis of Endosperm Color

Quantitative trait loci analysis was performed for endosperm color by SMA, CIM, and multiple trait-CIM. Single marker analysis for candidate genes was particularly important to test the hypothesis that they were directly responsible for differences in color. Out of the nine carotenoid candidate genes evaluated, *Ccd1a* was marginally significant (at $p \leq 0.05$) and *Psy3* was highly significant ($p \leq 0.0001$) for color (not corrected for multiple comparisons) (Table 4a).

CIM for endosperm color identified four QTL on chromosomes 1, 2, and 7, explaining 3.13 to 5.65% of the variance conditional on the background markers used as cofactors (Fig. 2, Table 5). Phenotypic values were averaged over locations, since analyses performed for each location separately showed coincidence of QTL positions. QTL on chromosomes 1 and 2 showed positive additive effects, meaning that, as expected, the yellow endosperm parent (KS115) allele contributed to an increase in intensity of the yellow color. The fourth QTL on chromosome 7 had a negative additive effect, with the KS115 allele reducing the level of yellow color. None of these four QTL co-localized with any of the nine carotenoid candidate genes mapped in this study.

Using multiple trait-CIM, eleven QTL were significant for endosperm color. Two of them were not previously identified by CIM but co-localized with carotenoid QTL (*Co-6.1* and *Co-10a.1*), three of them confirmed the color QTL previously identified by CIM (*Co-1.4*, *Co-2.2,* and *Co-7.1*), and six spanned genomic regions in which no other QTL were found (*Co-1.3*, *Co-1.5*, *Co-3.1*, *Co-5.1, Co-8.1,* and *Co-10b.1*) (Fig. 2). Only *Co-1.3* was localized in a region where a carotenoid candidate gene was mapped (*Ccd1a*), confirming results from single marker analysis (Table 4a). LOD scores and additive effects are summarized in Table 6.

QTL Analysis of Individual Carotenoid Compounds

As with endosperm color, SMA was performed for all markers but was particularly important to test carotenoid candidate genes as putative genetic components affecting carotenoid accumulation in sorghum endosperm. Six candidate genes were significantly associated with lutein (not corrected for multiple comparisons): *Crtrb1* at the 5% level and *Ccd1a, Vp14, Lcye, Psy3,* and *Psy1* at the 1% level. For zeaxanthin and β-carotene content only *Pds* and *Psy3* were significant (Table 4b).

Lutein, zeaxanthin, and β-carotene were evaluated by CIM using individual phenotypic values from each location. Since this analysis showed no co-localization of lutein QTL from College Station and Manhattan, values were not averaged over locations.

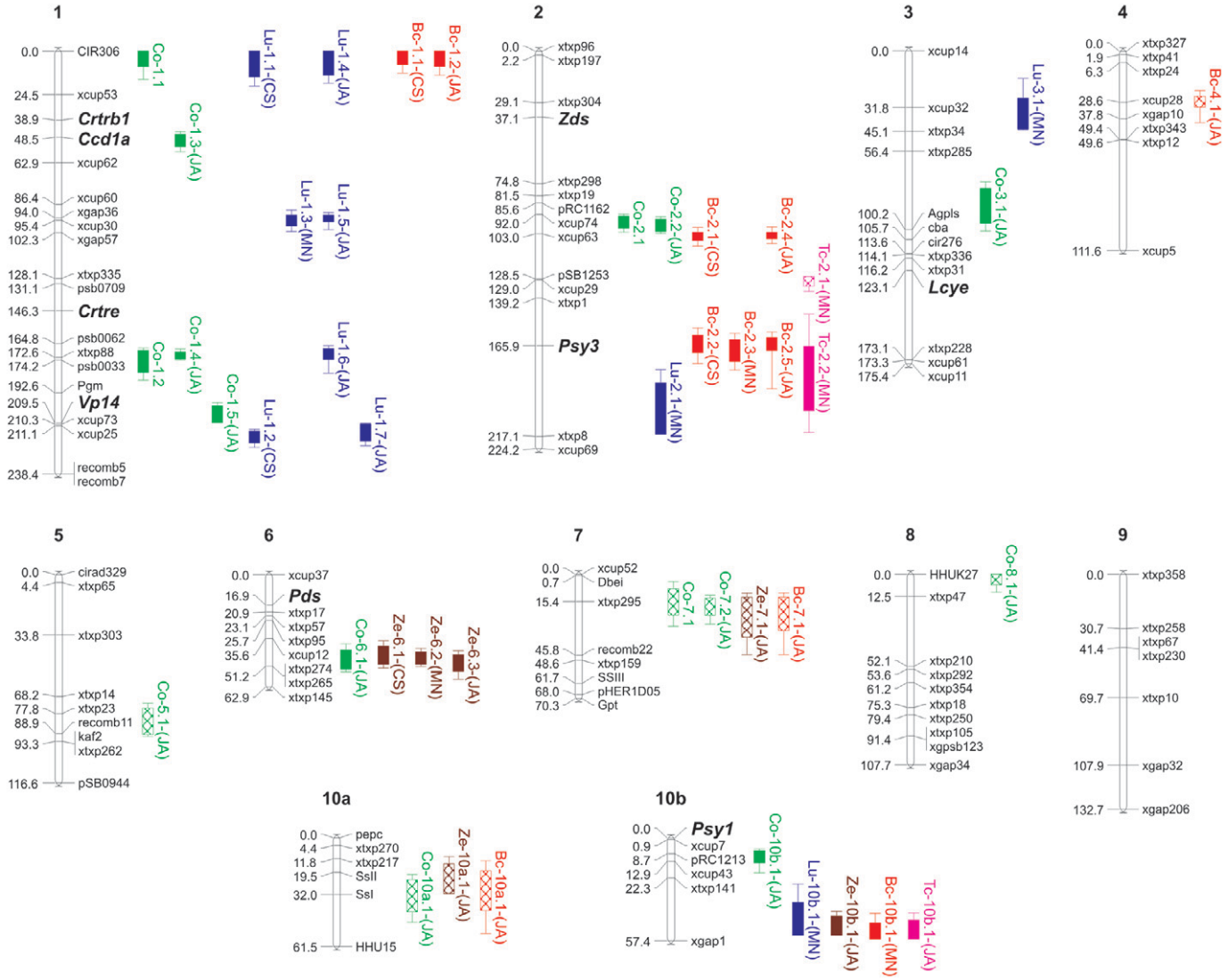

Figure 2. Genetic map and QTL summary for color and carotenoid content in sorghum endosperm. QTL are represented by bars (1-LOD interval) and extended lines (2-LOD interval). Solid bars represent QTL with positive additive effects and diagonal hatch bars represent QTL with negative additive effects in all cases referring to the KS115 allele at that particular QTL. Color codes indicate the trait: green for "color," blue for "lutein," brown for "zeaxanthin," red for "β-carotene," and pink for "total carotenoids." QTL names also indicate trait: Co (color), Lu (lutein), Ze (zeaxanthin), Bc (β-carotene), and Tc (total carotenoids). In parenthesis, either location or analysis used to detect that QTL is specified: CS for College Station, MN for Manhattan, and JA for joint-analysis using multiple trait-CIM. Carotenoid candidate genes are shown in bold. Figure prepared with MapChart 2.2. (R.E. Voorrips. Plant Research International, Wageningen, The Netherlands).

Two QTL were identified for lutein concentration in College Station and four for Manhattan (Fig. 2). Both QTL for lutein in College Station were mapped to chromosome 1, explained large proportions of the phenotypic variance and had positive additive effects (Table 5). The four lutein QTL identified with data from Manhattan were on chromosomes 1, 2, 3, and 10b. The percentage of the variance explained by each QTL varied from 7.13 to 28.5% and they all had positive additive effects (Table 5). None of the lutein QTL from College Station and Manhattan co-localized (Fig. 2).

Only one QTL for zeaxanthin concentration was identified in each location and they co-localized on chromosome 6. Both had very large additive effects and explained large proportions of the variance (Table 5).

Three QTL were found for β-carotene in College Station on chromosomes 1 and 2 (*Bc-1.1*, *Bc-2.1,* and *Bc-2.2*). All of them had positive additive effects and explained between 8 and 11.8% of the phenotypic variance. Two β-carotene QTL were identified in Manhattan (*Bc-2.3* and *Bc-10b.1*), also having positive additive effects and explaining 9.84 and 15.15% of the phenotypic variance (Table 5). *Psy3* gene was significantly associated with β-carotene QTL from both locations (*Bc-2.2* and *Bc-2.3*).

Multiple trait-CIM was used to test QTL identified by CIM and also to evaluate the possibility of finding new QTL, due to the higher detection power. For every trait, values from each location were individually used and analyzed as correlated traits. Four loci were identified for lutein, all of them in chromosome 1 and they co-localized to previously

identified QTL (Table 6). *Lu-1.4*, *Lu-1.5,* and *Lu-1.7* were in similar genomic regions where either College Station or Manhattan QTL for lutein were identified. *Lu-1.6* co-localized with color QTL. All four had positive additive effects and confirmed previously identified loci (Table 6, Fig. 2).

Four genomic regions were associated with zeaxanthin variation using multiple trait-CIM. Two of them (*Ze-6.3* and *Ze-10b.1*) co-localized with carotenoid QTL previously identified using CIM. However, two new regions were found associated with zeaxanthin on chromosomes 7 and 10a. *Ze-7.1* co-localized with color QTL (*Co-7.1* and *Co-7.2)* and a β-carotene QTL (*Bc-7.1*). *Ze-10a.1* was detected in the same genomic region as a β-carotene QTL (*Bc-10a.1*) and a color QTL (*Co-10a.1*) that were found using multiple trait-CIM. All of them had negative additive effects (Table 6, Fig. 2).

When β-carotene was analyzed by multiple trait-CIM, a total of six QTL were detected (Table 6). Two QTL were found on chromosomes 4 and 10a, where no previous QTL were identified using CIM. They both had negative additive effects and *Bc-10a.1* co-localized with *Ze-10a.1* and *Co-10a.1*. Three QTL (*Bc-1.2*, *Bc-2.4,* and *Bc-2.5*) confirmed β-carotene loci detected using CIM. The QTL identified on chromosome 7 (*Bc-7.1*) was found in the same position as color and zeaxanthin QTL (*Co-7.1*, *Co-7.2,* and *Ze-7.1*), all with negative additive effects (Table 6, Fig. 2).

QTL Analysis of Total Carotenoid Content

SMA showed there was a significant association of *Pds* ($p \leq 0.05$) and *Psy3* ($p \leq 0.001$) with total carotenoid content (not corrected for multiple comparisons) (Table 4c).

When levels of total carotenoids in the endosperm were analyzed by CIM using individual phenotypic data per location, QTL were found only in Manhattan. *Tc-2.2* had a positive additive effect, which explained a large proportion of the phenotypic variance (20.12%), and had large confidence intervals that overlap with β-carotene and lutein QTL (Table 5, Fig. 2).

Only one QTL was identified using "multiple trait-CIM" (Table 6). *Tc-10b.1* co-localized with QTL for lutein and β-carotene concentration in Manhattan *(Lu-10b.1, Bc-10b.1*), as well as with a QTL for zeaxanthin (*Ze-10b.1*) (Fig. 2).

DISCUSSION

Lutein, zeaxanthin, and β-carotene were the major carotenoid compounds varying in this sorghum RIL population, with zeaxanthin being on average the most abundant. The carotenoid profile described here is in agreement with the only recent report on carotenoid content of sorghum endosperm (Kean et al., 2007). Heritability values for carotenoids and endosperm color were high, a result consistent with previous data from maize (Wong et al., 2004). This important genetic characteristic indicates the likely success of a breeding program targeted to improve carotenoid concentration in sorghum grain.

Using CIM, we have identified four QTL for endosperm color, two QTL for lutein in College Station and four QTL for lutein in Manhattan. Only one QTL was observed for zeaxanthin in both locations; these QTL had major effects and explained large proportions of the phenotypic variance.

Although β-carotene, the compound with highest provitamin A activity, was at the lowest concentration, the level of variation allowed for identification of several major QTL.

All five β-carotene QTL identified using CIM might be important genomic regions for marker-assisted selection (MAS) in a breeding program to improve provitamin

Table 4. Single marker analysis for carotenoid candidate genes (phenotypic values averaged over both locations).

a: for endosperm color (measured as b value)

Candidate gene	Chromosome	LR	F (1,n–2)	Pr (F)
Crtrb1	1	2.759	2.753	0.098
Crtre	1	1.475	1.469	0.226
Ccd1a	1	6.288	6.312	0.013
Vp14	1	0.286	0.285	0.594
Zds	2	1.061	1.056	0.305
Psy3	2	22.48	23.16	0.000
Lcye	3	2.878	2.872	0.091
Pds	6	1.873	1.867	0.173
Psy1	11	1.801	1.795	0.181

b: for lutein, zeaxanthin, and β-carotene concentration

Candidate gene	Lutein		Zeaxanthin		β-carotene	
	F(1,n–2)	Pr (F)	F(1,n–2)	Pr (F)	F(1,n–2)	Pr (F)
Crtrb1	4.770	0.031	0.012	0.911	0.205	0.652
Crtre	0.004	0.948	1.920	0.168	0.002	0.965
Ccd1a	8.260	0.005	0.474	0.493	0.629	0.429
Vp14	8.482	0.004	0.520	0.472	0.715	0.399
Zds	0.251	0.617	0.012	0.914	1.502	0.223
Lcye	6.994	0.009	0.228	0.634	0.400	0.528
Psy3	7.107	0.009	6.976	0.009	47.79	0.000
Pds	0.603	0.439	7.921	0.006	12.175	0.001
Psy1	9.286	0.003	0.796	0.374	3.139	0.079

c: for total carotenoids

Candidate gene	Chromosome	LR	F (1,n–2)	Pr (F)
Crtrb1	1	0.534	0.527	0.469
Crtre	1	0.945	0.933	0.336
Ccd1a	1	2.185	2.169	0.143
Vp14	1	0.738	0.728	0.395
Zds	2	0.129	0.127	0.722
Psy3	2	11.08	11.40	0.001
Lcye	3	0.185	0.182	0.671
Pds	6	6.595	6.663	0.011
Psy1	10b	3.142	3.131	0.079

Table 5. QTL for color, lutein, zeaxanthin, and β-carotene using CIM.

Trait	QTL name	Location	Chr.	LOD score	QTL position (cM)			Additive effect	PVE (%)
					Peak (cM)	1 LOD	2 LOD		
Color†	*Co-1.1*	–	1	3.598	0	0–8.8	0–16.0	0.717	3.145
	Co-1.2	–	1	5.304	172.6	168.8–181.1	167.3–185.4	0.774	4.602
	Co-2.1	–	2	4.069	96	92.9–100.0	91.9–102.0	0.898	5.653
	Co-7.1	–	7	3.317	14.7	8.2–23.1	4.2–29.4	–0.564	3.131
Lutein	*Lu-1.1*	CS	1	4.846	6	0.0–14.6	0.0–19.7	4.535	17.9
	Lu-1.2	CS	1	5.402	215.1	213.2–220.8	214.3–223.4	7.472	26.4
	Lu-1.3	MN	1	3.414	94.0	92.4–98.5	89.6–101.6	3.572	7.13
	Lu-2.1	MN	2	5.940	203.9	186.9–215.9	179.5–215.9	6.712	28.5
	Lu-3.1	MN	3	5.284	39.8	26.7–44.0	15.6–44.3	4.495	13.7
	Lu-10b.1	MN	10b	3.467	52.3	36.0–54.3	25.7–54.3	4.301	10.7
Zeaxanthin	*Ze-6.1*	CS	6	5.243	45.6	40.0–50.3	37.0–52.2	12.50	18.18
	Ze-6.2	MN	6	8.056	47.6	43.3–50.3	41.1–51.0	10.51	25.4
β-carotene	*Bc-1.1*	CS	1	4.677	0.0	0.0–7.5	0.0–12.5	1.045	8.0
	Bc-2.1	CS	2	6.304	103.0	102.0–106.7	99.4–109.8	1.182	11.8
	Bc-2.2	CS	2	6.118	165.2	160.0–170.0	156.0–176.1	1.179	11.6
	Bc-2.3	MN	2	7.851	165.9	162.6–174.9	159.1–179.8	1.367	15.15
	Bc-10b.1	MN	10b	5.02	56.3	47.5–56.3	42.1–56.3	1.14	9.84
Total carotenoids	*Tc-2.1*	MN	2	3.738	128.5	127.0–132.2	127.0–135.4	–9.003	7.72
	Tc-2.2	MN	2	3.68	183.9	166.2–202.7	148.3–214.9	14.021	20.12

†Color QTL were analyzed using averaged values from both locations and reps. CS, College Station, Tx; MN, Manhattan, KS.

Table 6. QTL for color, lutein, zeaxanthin, β-carotene, and total carotenoids using multiple trait-CIM.

Trait	QTL name	Chr.	LOD score	Peak position cM	Additive effect
Color	*Co-1.3*	1	4.9	48.5	0.506
	Co-1.4	1	9.1	172.6	0.989
	Co-1.5	1	5.4	204.6	0.857
	Co-2.2	2	11.3	98.0	1.266
	Co-3.1	3	5.9	84.4	1.351
	Co-5.1	5	3.7	85.8	–0.619
	Co-6.1	6	5.0	49.6	0.589
	Co-7.2	7	9.0	15.4	–1.074
	Co-8.1	8	5.1	0.0	–0.808
	Co-10a.1	10a	7.6	29.5	–1.033
	Co-10b.1	10b	4.4	8.7	0.810
Lutein	*Lu-1.4*	1	5.3	4.0	4.301
	Lu-1.5	1	4.3	94.0	3.164
	Lu-1.6	1	4.2	170.8	3.243
	Lu-1.7	1	4.5	215.1	6.621
Zeaxanthin	*Ze-6.3*	6	10.1	49.6	10.982
	Ze-7.1	7	4.3	15.4	–8.481
	Ze-10a.1	10a	4.3	23.5	–8.152
	Ze-10b.1	10b	5.8	48.3	9.410
β-carotene	*Bc-1.2*	1	4.8	0.0	0.943
	Bc-2.4	2	6.6	103.0	1.279
	Bc-2.5	2	8.2	165.2	1.693
	Bc-4.1	4	5.6	28.6	–1.502
	Bc-7.1	7	4.0	15.4	–1.267
	Bc-10a.1	10a	5.2	27.5	–1.472
Total carotenoids	*Tc-10b.1*	10b	7.0	56.3	16.055

A content in sorghum used for human consumption. Two of the QTL from College Station (*Bc-1.1 and Bc-2.1*) co-localized to color QTL, which may simplify the selection process in a breeding program, if color can be reliably used instead of measuring β-carotene concentration. The QTL from Manhattan found on chromosome 10b (*Bc-10b.1*), spanned a region where other QTL for lutein, zeaxanthin, and total carotenoids were identified. Therefore, this region might be an important target for increasing total carotenoid content since it affects multiple compounds with positive and large effects. However, the β-carotene QTL identified close to *Psy3* might be the most significant QTL to consider when breeding for high provitamin A content, since for a MAS scheme it is desirable to select markers significant across environments and β-carotene QTL from both locations were identified in this region. Additionally, these QTL explained large proportions of the phenotypic variance and were close to a carotenoid candidate gene (*Psy3*). If in fact, as is suggested by this research study, *Psy3* is the genetic cause underlying the β-carotene QTL, marker-assisted selection would be very efficient since it could target the actual gene responsible for the phenotypic variation.

QTL for total carotenoid concentration were identified only for Manhattan when CIM analysis was used. *Tc-2.2* slightly overlapped with β-carotene QTL and mapped close to *Psy3*. When using multiple trait-CIM, *Tc-10b.1* was identified overlapping with several other QTL for lutein, β-carotene, and zeaxanthin. The fact

that one locus is significant for multiple carotenoid compounds as well as for total concentration has been previously described (Wong et al., 2004) and this type of QTL, affecting multiple carotenoids, could be valuable if the breeding objective is to simultaneously increase several compounds since they all have beneficial health effects.

We propose that QTL identified in this study should be tested and validated in other environments and evaluated using other populations. This way the large effects of QTL could also be confirmed, since the present value might have an upward bias due to the moderate size of our population (Melchinger et al. (2004) and Beavis (1998).

Considering that the present QTL study for different carotenoid compounds was performed in a selected subset of RILs from the original population of 312 lines and that some markers exhibited segregation distortion, we cannot rule out the possibility that we did not have enough power to detect QTL with minor effects that may have contributed to the trait variation.

In general, multiple trait-CIM was a useful method to increase detection power and confirmed results used with other methods. Validation of QTL in other locations could also be useful to confirm the QTL exclusively identified by multiple trait-CIM, such as the ones on chromosome 7 and 10a.

Endosperm color proved to be a valuable trait as an indicator of carotenoid concentration, since correlations were positive and significant with the three compounds and total carotenoids, as expected based on other species (Shewmaker et al., 1999; Paine et al., 2005; Thorup et al., 2000; Li et al., 2001; Lu et al., 2006). However, this result contradicts the low correlations between color and carotenoids found in maize (Harjes et al., 2008), a discrepancy that might be partly explained by the difference in methods used in the two studies. The pericarp removal and use of a colorimeter might be a more accurate way to measure color than a visual scale such as the one used by Harjes et al. (2008). Regardless of the low percentage of the variance explained by the four color QTL obtained by CIM, all of them co-localized with QTL for individual compounds. The correlation between color and zeaxanthin was the highest, reflected by the co-localization of all zeaxanthin QTL with color QTL except for *Ze-10b.1*. In general, we can conclude that endosperm color, measured with an optical system such as the one used in this study, could be a cheaper, faster, and useful trait to use under certain breeding conditions in which HPLC analysis of a large number of samples could not be feasible.

Nine candidate genes involved in carotenoid synthesis or degradation were mapped to test the hypothesis that any of them could be responsible for variation in carotenoid concentrations in the endosperm. Some candidate genes, particularly *Psy1* and *Zds*, have been reported as major genes responsible for increasing carotenoid concentrations. They were mapped under major QTL and linked to carotenoid mutants in several species (Wong et al., 2004; Paine et al., 2005; Thorup et al., 2000; Pozniak et al., 2007). Additionally, *Psy1* polymorphisms have been associated with the yellow/white endosperm phenotype in maize (Palaisa et al., 2003). In our study, several candidate genes showed significant association with the traits by SMA but results related to phytoene synthase genes are not in agreement with conclusions from other species. *Psy1* gene was not associated to either color or any carotenoid QTL detected using CIM or multiple trait-CIM. We have independently identified a new phytoene synthase gene (*Psy3*), which corresponds to the *Psy3* gene identified by Li et al. (2007b). In our study, *Psy3* was significantly associated with β-carotene concentration and endosperm color by SMA, and it was located in the genomic region where β-carotene QTL were detected. Even though there are no published studies providing either functional or expression information about this new *Psy3* gene in sorghum, results from maize showed that *Psy3* was highly expressed in roots and embryos, particularly under abiotic stress conditions, but expressed at low levels in endosperms (Li et al., 2007b). The fact that neither of the two sorghum phytoene synthase genes investigated in this study performed as expected on the basis of information from other species, raises the interest of developing further experiments to elucidate expression levels in different tissues and between yellow and white sorghum endosperms.

We are currently evaluating the association of variation in twelve candidate genes (including *Psy3*) with carotenoid accumulation in a diverse set of yellow and white endosperm sorghums, a study that might confirm the results reported here regarding the role of *Psy3* in carotenoid accumulation in sorghum endosperms. The type of molecular markers used to construct our genetic map could be anchored to a physical map; so as the sorghum genome sequencing project progresses, we might be able to identify other candidates physically located in the genomic regions where different carotenoid QTL were identified.

Our research is the first report of QTL for carotenoid content in sorghum grains. We proved that there is significant phenotypic variation for the trait and that genetic mechanisms are mainly responsible for that variation. The concentrations of carotenoids presented here are four to ten times lower (depending on the compound) than concentrations in common maize lines such as B73 (data not shown), a result in agreement with previous studies (Kean et al., 2007). However, this population was not developed with the goal of increasing carotenoids in sorghum, but rather to evaluate the genetic variation that controls carotenoid accumulation in sorghum endosperms. Even though β-carotene content in this population was low and would not be sufficient to cover daily requirement of vitamin A, several major QTL with

large effects were identified, which could be a starting point for breeding high-provitamin A sorghums. Increasing the content of other carotenoids, such as lutein and zeaxanthin, would also have a positive impact to alleviate some micronutrient deficiency problems, since they can help increase iron absorption (Garcia-Casal, 2006). We have detected several QTL related to quantitative variation of these two compounds, one of them simultaneously affecting lutein and zeaxanthin. Considering that sorghum is the main staple crop in a vast geographic area where vitamin A deficiency is an important health concern and where no other crops can be produced due to limited environmental conditions, it is worth the effort to continue studying the genetic control of carotenoid biosynthesis in sorghum endosperm and begin breeding for high-provitamin A content.

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