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ORIGINAL PAPER

Using molecular markers to identify two major loci controlling carotenoid contents in maize grain

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Abstract Maize is an important source of pro-vitamin A; β-carotene, α-carotene and β-cryptoxanthin, and the non-pro-vitamin A carotenoids including lutein and zeaxanthin. In the present study, a recombinant inbred (RI) population with 233 RI lines derived from a cross between By804 and B73 was employed to detect QTL for these nutritionally important components in maize grain. High Performance Liquid Chromatography was used to measure amounts of individual carotenoids over 2 years. A genetic linkage map was constructed with 201 molecular markers. In all, 31 putative QTL including 23 for individual and 8 for total carotenoids were detected on chromosome(s) 1, 3, 5, 6, 7, 8 and 10. The notable aspect of this study was that much of the phenotypic variation in contents of carotenoids could be explained by two loci (*y1* and *y9*), and the QTL for carotenoids elucidated the interrelationships among these compounds at the molecular level. A gene targeted marker (Y1ssr) in the candidate gene phytoene synthase 1 (*psy1*) tightly linked to a major QTL explaining 6.6–27.2% phenotypic variation for levels of carotenoids was identified, which may prove useful to expedite breeding for higher level of carotenoids in maize grain. This functionally characterized gene (*psy1*) could also be exploited for further development of functional marker for carotenoids in maize. The QTL cluster located at *y9* locus may also be used for pyramiding favorable alleles controlling contents of carotenoids from diverse maize germplasm.

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Introduction

Carotenoids are natural plant pigments that occur widely in plants. These are an important source of vitamin A and antioxidants that are essential for human consumption. In plants, carotenoids play a crucial role in photosynthesis, membrane stability, growth and development. By imparting varied colors to flower and fruits, carotenoids provide distinct characteristics to plants and may also participate in enhancement of pollination and seed dispersal under different ecosystem (Hirschberg 2001). Apart from pro-vitamin A activity, recent epidemiological studies have demonstrated several essential biological functions of carotenoids in human health, particularly in reducing the risk of degenerative diseases such as cardiovascular, cancers, cataract and macular degeneration (Fraser and Bramley 2004). Carotenoids can be classified into two groups on the basis of retinal structure, i.e. pro-vitamin A and non-pro-vitamin A carotenoids. Pro-vitamins A serves as a dietary source of vitamin A because it can be either enzymatically cleaved to yield either one molecule each of α-carotene and β-cryptoxanthin or two β-carotene molecules of retinal. On the other hand, non-pro-vitamin A carotenoids (lutein and zeaxanthin) acts as an antioxidants and together with pro-vitamin A carotenoids, these have been found to have several beneficial effects on human health. Vitamin A deficiency (VAD) continues to be major public health problem that affects more than 100 million children residing in 118 countries at the risk of blindness or death (UNICEF 2005). In long term,

biofortication of major staple food crops with enhanced level of carotenoids is the only feasible way to cope with VAD in rural areas of developing countries, since this will better ensure the targeting and compliance.

Carotenoids have been shown to possess complex heredity and hence are difficult to be genetically manipulated using normal breeding methods. Among cereals, maize is the only major crop that contains appreciable amount of carotenoids (Wurtzel 2004). Though the upstream and downstream regulation of carotenoids biosynthesis in maize endosperm is yet not fully characterized, some genes namely, phytoene synthase (*psy*1, 6.01 bin, Buckner et al. 1990, 1996; *psy*2, 8.07 bin, Gallagher et al. 2003), phytoene desaturase (*pds*, 1.02 bin, Li et al. 1996; Hable et al. 1998), zeta (ζ)-carotene desaturase (*zds*, 7.02 bin, Luo and Wurtzel 1999; Matthews et al. 2003) and lycopene beta (*β*)-cyclase (*lcyB*, 5.04 bin, Singh et al. 2003) that are involved in this pathway have been cloned and mapped. More recently, in maize a *pale yellow9* (*y9*) locus has been characterized that catalyzes the conversion of phytoene desaturase (*PDS*) product (9,15,9′-tri-*cis*-*ξ*-carotene) into zeta carotene desaturase (*ZDS*) substrate (9,9′-di-*cis*-*ξ*-carotene) (Li et al. 2007). This study thus helped in further elucidation of the carotenoid biosynthetic pathway in plants. In future, cloning of *y9* locus in maize will have direct impact on better understanding of plant carotenoids biosynthesis.

Most of the previous studies on maize carotenoids have been limited to phenotypic variation for these compounds, and only few studies involving QTL analysis have been conducted. Although, Wong et al. (2004) could identify some QTL for nutritionally important carotenoids, the results were based on DNA polymorphism generated using segregating populations. In the present communication, we report results from our experiments to localize the quantitative trait loci (QTL) controlling contents of carotenoids, and to estimate their individual effects on carotenoids level through SSR markers utilizing a large recombinant inbred (RI) population. The focus is on the relatively important QTL for carotenoids with the candidate gene targeted markers (GTM) from carotenoid biosynthetic pathway in maize, and to evaluate the potential for functional markers development for biofortication of maize in the breeding program.

Materials and methods

Experimental material and field evaluation

A total of 233 recombinant inbred lines (RILs) used in the present study were derived from the cross between By804 and B73. The advanced generations (F_6 and F_7) of the RILs along with both the parents were grown in a randomized complete block design with two replications at the agronomy farm, China Agricultural University, Beijing during summer 2004 and 2005. Each genotype was grown in a row length of 4 m, spaced 0.67 m apart at a planting density of 45,000 plants/ha. Recommended cultural practices were followed. Each genotype was self-pollinated to avoid xenia effects and after maturity individual ear in each line was harvested, separately. Harvested grains were bulked within lines at shelling and stored in darkness at 4°C until carotenoids extraction to avoid loss of carotenoids (Quackenbush 1963). All the selfed ears in a line were bulked to get a representative sample from each genotype for data collection.

Carotenoids standards

The standards of *β*-carotene and α-carotene were extracted from sweet potato and carrot, respectively, whereas the kernels of fresh maize (about 40 days after pollination) were used as a source of *β*-cryptoxanthin, lutein and zeaxanthin following open column chromatography (Rodriguez-Amaya and Kimura 2004). Finally, each carotenoid standard was purified and confirmed in high performance liquid chromatography (HPLC) by observing the single peak for the corresponding carotenoid in chromatogram, and the photodiode array spectrum (PAS) at the ascending and descending slopes and at the maximum. All the carotenoids standard were more than 95% pure, except lutein that showed 92% purity. Carotenoid standards and samples were prepared in a dark room, since these are highly sensitive to light, heat and oxygen (Weber 1987).

Carotenoids extraction and estimation

In a 50 ml measuring cylinder containing about 35 g maize grains from each line were ground in FW100 Stein mill for 2 min and stored at 4°C before extraction of carotenoids. The carotenoids extraction and measurement procedure described by Kurilich and Juvik (1999) and later modified by Egesel et al. (2003a) were followed. A 20 µl volume of each sample was manually injected into the HPLC system of Shimadzu Corporation (Kyoto, Japan) attached with a photodiode array detector (PDA). *β*-carotene, α-carotene, *β*-cryptoxanthin, lutein and zeaxanthin peaks were detected at 450 nm with 1.2 nm wavelength resolutions. The total carotenoids were computed by adding all the individual carotenoids in a sample, while individual carotenoids were detected by standard regression with external standards as calculated by the Shimadzu LC software. Concentration of each standard extracted in our laboratory was determined with the help of spectrophotometer using Lambert-Beer Law (Kurilich and Juvik 1999; Rodriguez-Amaya and Kimura 2004). Six dilutions of each carotenoid were used

to make the standard curve. YMC® carotenoid C^{30} column (5 µm, 4.6 × 250 mm; Waters Chromatography, Milford, MA), especially manufactured for carotenoids detection was used to estimate the individual carotenoid in each sample. An external column heater was set at a column temperature of 30°C to avoid erratic retention time of carotenoids among the samples.

Genotypic data and genetic linkage maps

Genomic DNA was extracted from young leaf tissue following cetyl trimethyl ammonium bromide (CTAB) method (Murry and Thompson 1980). SSR markers were selected from public maize database (http://www.maizegdb.org) for construction of genetic map. The basic SSR procedure was followed: template DNA 50 ng, primers 0.67 µM, 10 × reaction buffer 1×, $Mgcl_2$ 2.5 mM, dNTPs 0.2 mM, Taq DNA polymerase 0.5 U, made the final volume of 15 µl with deionized double distilled water, and finally covered with one drop of mineral oil. The PCR reaction conditions were set as: denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, elongation at 72°C for 1.5 min, with final extension at 72°C for 5 min and stored in refrigerator at 4°C. Amplified product was separated on 6% polyacrylamide gel electrophoresis (PAGE) and visualized by silver-staining (Xu et al. 2002).

A total of 201 molecular markers including 179 SSR, 8 CAPS and 14 STS were used for constructing a genetic linkage map, covering 1656.5 cM length of all the chromosomes. A set of 179 SSR markers comprising 178 random DNA markers and one gene targeted SSR marker derived from carotenoid biosynthetic pathway gene (*psy*1) representing *y1* locus were assigned. These exhibited clear-cut polymorphism between two parents By804 and B73. In addition, 22 PCR based markers (CAPS and STS) derived from 20 ESTs and two carotenoid biosynthetic pathway genes namely *zds* and *psy*2 were also used. Molecular linkage map for RIL population was constructed by using the MAPMAKER/EXP ver. 3.0 program (Lincoln et al. 1992). Linkage was inferred using a logarithm of odds (LOD) threshold of 3.0 and maximum distance between two loci of 50 cM for forming linkage group, the 'ripple' command was used for establishing most likely marker order.

Statistical analysis

Analysis of variance for carotenoid content and composition in each year as well as pooled analysis was performed using the "PROC GLM" procedure of SAS (SAS Institute, version 8.0, 1999) to determine variation between two years. The components of variance were estimated using a complete random effects model. To study the interrelationship among the carotenoid compounds, simple correlation coefficients were used in this experiment. The broad-sense heritability (h^2_B) was computed according to Knapp et al. (1985).

The composite interval mapping (CIM) method was employed for QTL mapping using Wincartographer 2.5 software (Zeng 1994). Model 6 of the Zmapqtl module of QTL Cartographer was used to detect QTL with scanning interval of 2 cM between markers and putative QTL with a window size of 10 cM. The number of marker cofactors for background control was set by forward–backward stepwise regression with five controlling markers. A genome-wide critical threshold value for the experiment-wise type I error rate (alpha = 0.05) was set for each trait independently, by 1,000 random permutations (Churchill and Doerge 1994). For main QTL effects, positive and negative signs of the estimates indicate that B73 and By804 contributed toward higher value alleles for the traits, respectively. The proportion of phenotypic variance explained by a single QTL was determined by the square of the partial correlation coefficient (R^2). Putative QTL for individual traits exceeded the threshold LOD value in joint analysis decided after permutation test were considered.

Results

Significant genetic differences among the two parents and RIL progenies for carotenoid content and composition were observed (Table 1). A wide range of variation in the traits suggested transgressive segregation and polygenic mode of inheritance. The combined mean values of RILs for β-carotene, α-carotene, β-cryptoxanthin, lutein, zeaxanthin and total carotenoids were 0.512, 0.546, 0.282, 7.354, 1.510 and 10.202 µg g^{-1}, respectively (Table 1). During 2005, carotenoids namely β-carotene, α-carotene, β-cryptoxanthin and zeaxanthin had higher values with mean 0.566, 0.597, 0.332 and 1.544 µg g^{-1} vis a vis 0.457, 0.494, 0.231 and 1.476 µg g^{-1} in 2004. In contrast, lutein and total carotenoids exhibited highest mean in 2004 with 8.020 and 10.679 µg g^{-1} vis a vis 6.687 and 9.726 µg g^{-1} in 2005, respectively. A relatively higher proportion of β-carotene, α-carotene, β-cryptoxanthin and zeaxanthin, and a relatively lower proportion of lutein were observed in 2005 (Table 1).

Broad-sense heritabilities $(h)^2_B$ for β-carotene, α-carotene, β-cryptoxanthin, zeaxanthin, lutein and total carotenoids were 94, 96, 86, 88, 84 and 85%, respectively (Table 2). A perusal of Table 2 indicates that there was significant and positive correlation among individual as well as total carotenoids within this set of investigated RILs population. The magnitudes of correlation of zeaxanthin with lutein and α-carotene were low, but statistically significant. The

Table 1 Variation of carotenoids content and composition (μg g^{-1} seed dry weight) in parental and 233 recombinant inbred lines grown during summer 2004 and 2005

Traits	Mean					Proportion to total carotenoids (%)				Range
	Parents		RILs			Parents		RILs		
	By804	B73	2004	2005	Combined	By804	B73	2004	2005	
β-Carotene	0.862 ± 0.056	0.360 ± 0.015	0.457 ± 0.043	0.566 ± 0.046	0.512 ± 0.031	6.40	3.65	4.28	5.82	0.052–2.040
α-Carotene	0.983 ± 0.047	0.652 ± 0.066	0.494 ± 0.043	0.597 ± 0.058	0.546 ± 0.036	7.30	6.62	4.63	6.14	0.015–1.589
β-Cryptoxanthin	0.281 ± 0.034	0.751 ± 0.046	0.231 ± 0.022	0.332 ± 0.041	0.282 ± 0.023	2.09	7.62	2.16	3.41	0.064–1.157
Lutein	9.927 ± 0.448	5.249 ± 0.378	8.020 ± 0.471	6.687 ± 0.588	7.354 ± 0.377	73.73	53.29	75.10	68.75	2.592–14.662
Zeaxanthin	1.411 ± 0.129	2.778 ± 0.763	1.476 ± 0.104	1.544 ± 0.155	1.510 ± 0.094	10.48	28.20	13.82	15.87	0.525–5.101
Total carotenoids	13.464 ± 0.698	9.850 ± 0.595	10.679 ± 0.458	9.726 ± 0.752	10.202 ± 0.440	–	–	–	–	3.651–18.273

Parental and combined mean values of RILs are pooled of 2004 and 2005 season

higher degree of positive correlation observed between individual carotenoids in this set of RILs population demonstrated the possibility of correlated response to selection.

QTL detection for carotenoids

After permutation test of pooled analysis, a total of 31 putative QTL including 23 for individual and 8 for total carotenoids were detected on the chromosome(s) 1, 3, 5, 6, 7, 8 and 10. A total of four QTL for each β-carotene and β-cryptoxanthin, five for each of α-carotene, zeaxanthin and lutein, and eight for total carotenoids with additive effect were found (Table 3, Fig. 1). It should be noted that most of the QTL (12 QTL) for individual as well as total carotenoids were located only on chromosome(s) 6 and 10. These included a major QTL on chromosome 6 with the largest effect (6.6–27.2%) on levels of individual as well as total carotenoids (Table 3, Figs. 1, 2a). The peak position of QTL for β-carotene and α-carotene, and zeaxanthin detected by CIM was mapped in the interval at 27.7 and 29.7 cM (YIssr–umc1595), respectively. On the other hand, the peak positions for β-cryptoxanthin, lutein and total carotenoids were mapped at 27.5 cM located between marker umc2313–YIssr on chromosome 6 (Table 3, Fig. 2a). The peak position of QTL detected for lutein and total carotenoids slightly shifted between position 27.5 and 27.7 cM during 2004 and 2005, respectively. Hence, the flanking markers of peak also changed from Y1ssr–umc1595 to umc2313–Y1ssr, although in pooled analysis the peak position of QTL detected for lutein and total carotenoids were mapped at 27.5 cM. The phenotypic variance explained (PVE) by these major QTL was 17.4, 6.6, 13.6, 13.9, 27.2 and 25.1% for β-carotene, α-carotene, β-cryptoxanthin, zeaxanthin, lutein and total carotenoids, respectively. The favorable alleles that possess additive effect were contributed from the parent By804. These were −1.35, −0.71, −0.51, −2.13, −1.01 and −1.32 for β-carotene, α-carotene, β-cryptoxanthin, zeaxanthin, lutein and total carotenoids, respectively (Table 3). In addition, a QTL for α-carotene accounting for 11.4% of the total phenotypic variation was also identified on chromosome 6 (112.1 cM) in the interval umc2162–phi29985 (Table 3, Fig. 1).

The QTL for all the carotenoids expect lutein detected in three adjacent intervals between 41 and 67 cM on chromosome 10 were clustered near one another (Table 3, Figs. 1, 2b). The interval umc1506–bnlg1028 showed the peak position of QTL for β-carotene and zeaxanthin at position 63.1 and 65.1 cM, respectively. While the interval umc2016–bnlg1655 and phi050–umc1367–umc2016 exhibited a QTL for α-carotene and β-cryptoxanthin at 44.8 and 41.1 cM position, respectively (Fig. 2b). As the peak position of QTL detected for β-cryptoxanthin slightly moved at position 41.8 cM in 2005, so the flanking marker thus changed

Table 2 Correlation coefficients among individual and total carotenoids (μg g^{-1} seed dry weight) in 233 RILs of maize derived from By804 × B73

	α-Carotene	β-Carotene	β-Cryptoxanthin	Lutein	Zeaxanthin	h^2_B(%)
α-Carotene	1					96
β-Carotene	0.369**	1				94
β-Cryptoxanthin	0.375**	0.339**	1			86
Lutein	0.407**	0.480**	0.383**	1		84
Zeaxanthin	0.133*	0.219*	0.744**	0.126*	1	88
Total carotenoids	0.490**	0.561**	0.560**	0.971**	0.737**	85

Significance of the correlation coefficients at the 5 or 1% level is indicated by * or **, respectively

from phi050–umc1367 to umc1367–umc2016. This cluster of putative QTL exhibited 16.3, 4.7, 5.8 and 16.6% phenotypic variation for β-carotene, α-carotene, β-cryptoxanthin and zeaxanthin content, respectively (Table 3). For these QTL except β-carotene, an increase in α-carotene, β-cryptoxanthin and zeaxanthin content were due to allelic contribution of the parent B73, as demonstrated by the positive value of the mean additivity at these QTL. At sub-threshold LOD value 2.70, a QTL for total carotenoids (67.0 cM) was identified at interval umc1506–bnlg1028, explaining 3.5% of the total phenotypic variation for the total carotenoids (Table 3). During 2004, a small peak of QTL at sub-threshold level 1.61 was also detected for lutein at position 67.0 cM, whereas in 2005 and in pooled analysis, this peak did not appear at this position (Fig. 2b).

In addition to chromosome(s) 6 and 10, chromosome 8 also carried an important QTL for β-cryptoxanthin and zeaxanthin within the interval umc1562–umc1141 explaining 4.9 and 5.8% of total phenotypic variation, respectively. A minor QTL for zeaxanthin clustering near 69.1 cM was also detected in interval phi115–phi10017–umc1735, which accounted for 3.8% of the phenotypic variation. The favorable alleles for β-cryptoxanthin and zeaxanthin were available in B73 (Table 3).

Some QTL with important effects were also detected for carotenoids on chromosome(s) 1, 3, 5 and 7 (Table 3, Fig. 1). Six QTL located on chromosome 1 for xanthophylls (one each for β-cryptoxanthin, lutein and zeaxanthin) and total carotenoids (3) had a PVE value ranging from 3.1 to 6.5%. The favorable alleles for putative QTL detected for β-cryptoxanthin and zeaxanthin came from parent B73, whereas By804 contributed the favorable allele for QTL associated with lutein (Table 3). Parent B73 also contributed the favorable alleles for QTL located on chromosome(s) 5 and 7 for β-carotene, lutein and total carotenoids at intervals umc1692–umc2373 and phi091–atf2–umc2332, respectively. Chromosome 3 had QTL for α-carotene in two separate intervals umc2408–bnlg197 and umc1399–phi046 with 12.0 and 18.8% PVE, respectively (Table 3, Fig. 1).

Mapping of the candidate gene targeted markers (GTM) and co-location of QTL for carotenoids

The Y1ssr marker situated in *psy*1, a prime gene of the carotenoid biosynthetic pathway located at *y1* locus on chromosome 6 was mapped at position 27.7 cM in this RIL population. The peak positions of major QTL for individual as well as total carotenoids were mapped within 2 cM of Y1ssr (Fig. 2a). The peak positions of QTL for β-carotene and α-carotene coincided with the position of Y1ssr marker and the peak positions for β-cryptoxanthin, lutein and total carotenoids were detected 0.2 cM away from the Y1ssr marker. Only the peak position of QTL associated with zeaxanthin was detected 2 cM away from Y1ssr marker (Table 3, Figs. 1, 2a). The candidate gene ζ-carotene desaturase (*zds*) located at *vp9* locus did not show any length polymorphism between the parents, but an SNP was detected; By804 parent had a guanine and the B73, an adenine. Consequently, B73 parent showed restriction site for endonuclease Bcl1 that mapped near marker phi034 on chromosome 7 (7.02 bin). In contrast, phytoene synthase 2 (*psy*2) exhibited 4 bp deletion in B73 and mapped on chromosome 8 (8.07 bin) near marker umc1268. The present investigation did not detect any QTL for carotenoids near *zds* and *psy*2 genes in this set of RILs population.

Discussion

In maize, carotenoids mainly occur in endosperm tissue of the grain (Steenbock and Coward 1927; Blessin et al. 1963b) and due to the triploid nature of endosperm; dosage effect plays an important role in carotenoids accumulation (Mangelsdorf and Fraps 1931; Egesel et al. 2003b) and it has complex inheritance. A RI population is an ideal material for studying the genetics of carotenoids accumulation, since in contrast to the segregating population, RI populations are genetically uniform and free from dominance, dominance × dominance and additive × dominance epistatic components of genetic variance, and these can be

Table 3 Individual and joint composite interval mapping results, estimated after permutation test for contents of carotenoids in 233 RILs of maize derived from By804 × B73

Traits (μg g^{-1} dry weight)	Chr.	Flanking markers	2004				2005				Pooled			
			P	LOD	*A*	R^2	*P*	LOD	*A*	R^2	*P*	LOD	*A*	R^2
β-Carotene	5	Umc1692–umc2373	80.2	3.35	0.65	4.33	78.2	2.94	0.59	3.07	80.2	3.01	0.61	3.6
	6	YIssr-umc1595	27.7	14.95	−1.37	18.13	27.7	13.99	−1.38	16.26	27.7	15.18	−1.35	17.4
	7	bnlg1792–phi091–atf2	66.1	3.66	0.64	3.98	64.2	3.07	0.67	4.00	66.1	3.05	0.56	3.1
	10	umc1506–bnlg1028	65.0	13.83	−1.43	18.32	65.0	13.83	−1.43	18.32	63.1	14.19	−1.28	16.3
α-Carotene	3	umc2408–bnlg197	144.0	4.93	−1.07	15.07	144.0[a]	2.57	−0.87	8.51	144.0	3.73	−0.98	12.0
	3	umc1399–phi046	163.5	8.10	−1.31	21.91	163.5	5.23	−1.17	15.0	163.5	6.95	−1.24	18.8
	6	Yissr–umc1595	27.7	3.53	−0.59	4.81	27.7	5.32	−0.82	7.7	27.7	4.76	−0.71	6.6
	6	umc2162–phi29985	112.1	4.46	0.92	11.83	112.1	4.05	0.97	10.9	112.1	4.22	0.93	11.4
	10	umc2016–bnlg1655	42.8	3.44	0.59	4.71	44.8[a]	2.83	0.62	4.4	44.8	3.10	0.60	4.7
β-Cryptoxanthin	1	bnlg2086–umc1988	102.3	3.11	0.27	4.16	102.3	3.71	0.34	4.30	102.3	3.48	0.28	4.1
	6	umc2313–YIssr	27.5	5.83	−0.38	7.95	27.5	14.31	−0.69	18.20	27.5	10.86	−0.51	13.6
	8	umc1562–umc1141	69.1[a]	2.28	0.22	2.9	69.1	4.86	0.39	5.69	69.1	4.09	0.31	4.9
	10	phi050–umc1367–umc2016	41.1	4.74	0.37	7.98	41.8	3.09	0.32	4.02	41.1	3.87	0.33	5.8
Zeaxanthin	1	phi30870–umc1553	210.2	3.05	1.52	6.29	210.2	3.45	1.45	6.49	210.2	3.41	1.39	6.1
	6	Yissr–umc1595	29.7	6.69	−1.98	10.33	29.7	11.02	−2.37	16.69	29.7	9.57	−2.13	13.9
	8	phi115–phi10017–umc1735	60.3	3.37	1.33	4.78	60.3	2.46	1.01	3.1	60.3	3.02	1.11	3.8
	8	umc1562–umc1141	69.1	4.85	1.63	6.88	69.1	3.54	1.23	4.46	69.1	4.61	1.39	5.8
	10	umc1506–bnlg1028	65.1	10.77	2.66	18.85	67.0	8.96	2.28	15.79	65.1	10.26	2.32	16.6
Lutein	1	umc1403–fad83	60.3[a]	2.07	−0.35	3.0	58.3[a]	2.33	−0.34	2.61	58.3	3.51	−0.39	4.0
	5	umc1447–umc1692–umc2373	82.2	3.45	0.47	5.41	76.9	3.16	0.43	4.17	80.2	3.74	0.44	5.3
	6	umc2313–Yissr–umc1595	27.5	13.81	−0.90	19.65	27.7	19.41	−1.09	26.27	27.5	20.03	−1.01	27.2
	7	phi091–atf2	66.1[a]	2.47	0.36	3.12	66.9[a]	2.43	0.35	2.74	66.1	4.97	0.46	5.8
	7	atf2–umc2332	78.9[a]	2.40	0.71	12.7	78.9	2.91	0.84	16.36	76.9	4.48	0.79	17.4
Total carotenoid	1	umc1403–fad83	58.3[a]	2.02	−0.42	2.35	58.3[a]	2.10	−0.42	2.17	58.3	3.15	−0.46	3.1
	1	umc2047–ols1	194.5[a]	2.19	0.47	3.12	194.5[a]	2.27	0.47	2.83	194.5	3.26	0.52	4.0
	1	phi30870–umc1553	208.2	3.36	0.65	5.94	206.2	3.98	0.65	5.41	208.2	4.27	0.65	6.5
	5	umc2115–umc1447	62.0[a]	2.62	0.63	5.51	62.0	3.23	0.72	6.44	60.0	3.42	0.62	5.8
	5	umc1692–umc2373	80.2[a]	2.61	0.51	3.67	78.2	4.42	0.62	4.80	78.2	4.19	0.54	4.4
	6	umc2313–Yissr–umc1595	27.5	16.22	−1.29	22.39	27.7	22.06	−1.53	28.25	27.5	20.17	−1.32	25.1
	7	bnlg1792–phi091–atf2	66.1[a]	2.80	0.49	3.36	64.2[a]	2.50	0.53	3.54	66.1	4.96	0.59	5.3
	7	atf2–umc2332	80.9	2.82	1.04	15.27	78.9[a]	2.54	1.07	14.51	80.9	5.02	1.21	22.3
	10	umc1506–bnlg1028	65.0[a]	2.10	0.46	2.93	69.0[a]	1.67	0.42	2.19	67.0[a]	2.70	0.48	3.5

Chr. Chromosome; *P* Peak position of QTL (cM); *LOD* Logarithm-of-odds; *A* Additive and R^2 Phenotypic variation explained by QTL (%), whereas positive and negative value indicates contribution of parent B73 and By804, respectively

[a] QTL with sub-threshold LOD score detected by permutation test

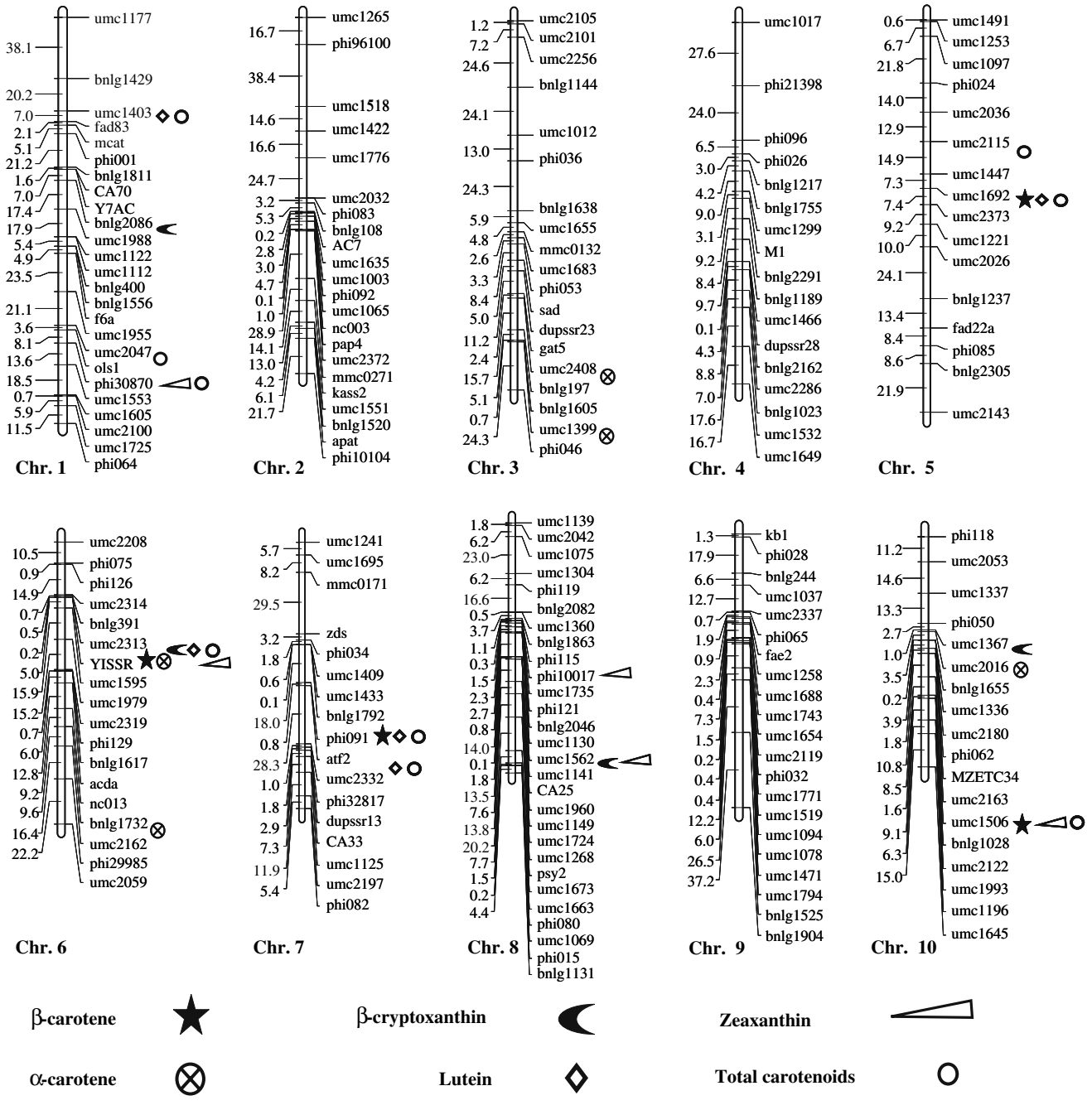

Fig. 1 Genetic linkage map and locations of putative QTL for carotenoids detected in 233 RILs derived from a cross between By804 and B73. The genetic distance between markers are given in cM (kosambi) and the symbols in linkage map indicate the position of QTL for respective carotenoid

evaluated at multi-locations in different years. Thus, these results in the present investigation with a RIL population provided more precise information about the genetic basis of carotenoids in maize.

Generally, yellow/orange maize grains possess lower level of pro-vitamin A as compared to non-pro-vitamin A carotenoids. The results from our RIL population showed lutein as a predominant carotenoid component. The transgressive segregation for contents of carotenoids in maize grain observed in the present study is in agreement with the results of Wong et al. (2004). In addition, significant genetic variation among RILs and higher heritability estimates $(h)^2_B$ for individual as well as total carotenoids (Table 2) could benefit breeding for higher level of these compounds in maize grain. The moderate to high heritability estimates for carotenoids have also been reported in the

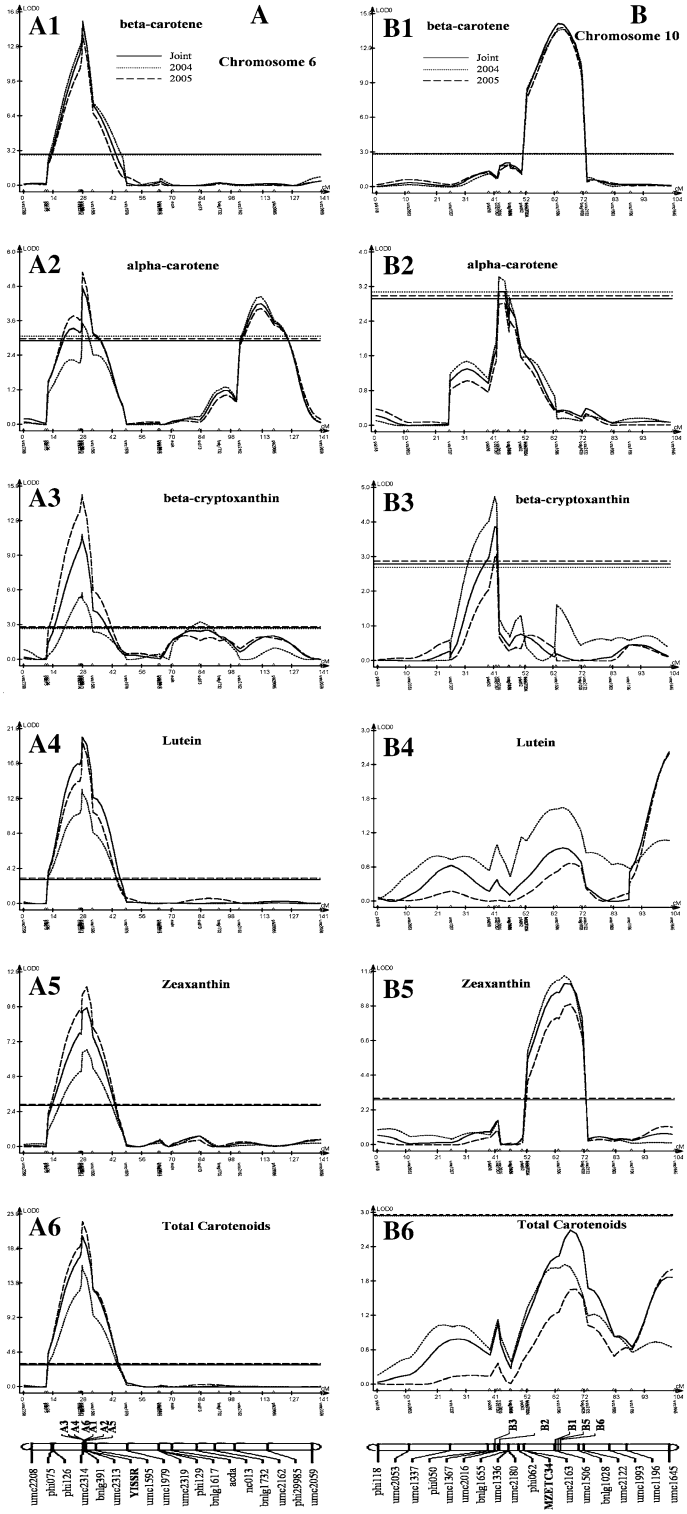

Fig. 2 QTL likelihood maps indicating LOD score, position of markers and major QTL detected on chromosome 6 and 10 for individual and total carotenoids. **a** and **b** represents chromosome 6 and 10, respectively; whereas *A1* and *B1*, *A2* and *B2*, *A3* and *B3*, *A4* and *B4*, *A5* and *B5* and *A6* and *B6* indicating LOD score, position of markers and major QTL for β-carotene, α-carotene, β-cryptoxanthin, lutein, zeaxanthin and total carotenoids, respectively

past for maize (Brunson and Quackenbush 1962; Blessin et al. 1963a; Wong et al. 2004), chickpea (Abbo et al. 2005) and carrot (Santos and Simon 2006)

The notable finding of the present study is that much of the phenotypic variation for levels of carotenoids could be explained by two loci located on chromosome(s) 6 and 10. Further, some QTL affected more than one carotenoid compounds. The detection of QTL associated with individual and total carotenoids also explains the molecular basis to the positive associations among individual carotenoids within this set of RIL population as reflected in congruence of specific loci on chromosome(s) 6 and 10. This could be

due to pleiotropy (*y1* locus) or tight linkage (QTL clusters at *y9* locus). This information is vital for the breeders as this opens up the possibility of simultaneous enhancement of different carotenoids.

Due to the complex inheritance of quantitative traits, the candidate gene approach is more amenable to QTL characterization than positional cloning or insertional mutagenesis (Pflieger et al. 2001). Although gene targeted marker (GTM) aided selection has advantages over random DNA marker aided selection, validation is required to confirm the presence of the target allele from candidate gene. Based on the knowledge of metabolic pathways, if gene targeted marker is associated with the QTL for concerned traits, candidate gene could be used for the development of functional markers (Andersen and Lübberstedt 2003). In present study, a major QTL at *y1* locus on chromosome 6 that accounted for 6.6–27.2% phenotypic variation for individual as well as total carotenoids was detected. The strong interactions of carotenoid biosynthetic pathway genes especially phytoene synthase gene with quantitative variation for carotenoids in maize grain observed in the present study was in substantial agreement with results from earlier studies in maize (Palaisa et al. 2003; Wong et al. 2004), solanaceae (Thorup et al. 2000), pepper (Huh et al. 2001), canola (Ravanello et al. 2003), tomato (Liu et al. 2003) and rice (Paine et al. 2005). In the current investigation, however, α-carotene that has half the β-carotene pro-vitamin A activity was also included in addition to the carotenoids that were studied by Wong et al. (2004). Thus, location of QTL for all individual as well as total carotenoids including α-carotene within 2 cM of Y1ssr marker in current investigation confirmed the allelic effect of *psy*1 gene on carotenoids accumulation in maize grain.

Another important locus on chromosome 10 exhibited association with cluster of QTL for carotenoids (Figs. 1, 2b). Five QTL one each for α-carotene, β-carotene, β-cryptoxanthin, zeaxanthin and total carotenoids were located within 15 cM of marker MZETC34/umc2163 (10.03 bin). At present, there is no report showing association of chromosome 10 with any known carotenogenic gene(s) in maize. However, *y9* locus associated with variation in carotenoids pigment in maize endosperm has been reported to be located on chromosome 10 (10.03 bin, http://www.maizegdb.org). Janic-Buckner et al. (2001) observed that the amount of carotenoids in maize endosperm tissue is drastically reduced in plants homozygous for *y*9. The role of maize *y*9 loucs for Z-ISO activity in carotenoid biosynthetic pathway has now been established (Li et al. 2007). The coincidence of bin (10.03 bin) location of marker MZETC34 with *y9* locus on chromosome 10, and the quantitative variation for carotenoids detected in the present investigation underlined the importance of this locus for carotenoids accumulation in maize grain. Thus, at molecular level (QTL analysis) first time the present investigation is demonstrating the importance of this locus (*y*9) for carotenoid accumulation in maize grain and the QTL cluster identified on chromosome 10 near marker MZETC34/umc2163 could lead to identification of the underlying genes at this locus for carotenoids pathway in maize grain.

Interestingly, favorable allele of QTL for only β-cryptoxanthin and zeaxanthin located on chromosome 8 were contributed by B73, indicating the presence of major loci associated with hydroxylation of carotene, as β-cryptoxanthin and zeaxanthin are the oxygenated derivatives of β-carotene and are produced from β-carotene with the activities of hydroxylases enzymes. The marker umc1562 in interval umc1562–umc1141 associated with QTL for β-cryptoxanthin and zeaxanthin was located on bin 8.05 of chromosome 8. Wong et al. (2004) also mapped QTL for xanthophylls (β-cryptoxanthin, zeaxanthin and lutein) at this region of chromosome 8 (8.05 bin) using $F_{2:3}$ and BC_1S_1 segregating populations and predicted that these QTL may influence the rate of the addition of the hydroxyl group to the β-ring of carotene in maize grain. In present study, phytoene synthase 2 (*psy*2) gene involved in carotenoids biosynthesis was mapped on chromosome 8 near marker umc1268, which is away from these QTL located on chromosome 8. Thus, only QTL for xanthophylls detected in the region (8.05 bin) of chromosome 8 in the present study and by Wong et al. (2004) controlled the isoprenoid pathway flux for xanthophylls and suggested the possible existence of major loci in this chromosomal region that influenced the rate of hydroxylation of carotenes in maize grain.

Another carotenoid biosynthetic pathway gene, *zds* mapped near marker phi034 (7.02 bin) on chromosome 7 was not found to be associated with any QTL for carotenoids in the present study. Contrary to this, Wong et al (2004) detected QTL for individual as well as total carotenoids within 4 cM of this gene in $F_{2:3}$ population. However, BC_1S_1 progenies did not allow detection of QTL for carotenoids near this gene. The result of the present study as well as those of Wong et al. (2004) suggested that carotenoid biosynthesis pathway genes namely phytoene desaturase (*pds*, 1.02 bin), lycopene β-cyclase (*lcyB*, 5.04 bin) and phytoene synthase 2 (*psy*2, 8.07 bin) cloned in maize, did not always associate with the quantitative variation for carotenoids in maize. This association thus seems to be genotype-dependent.

Carotenoids especially pro-vitamin A carotenoids in maize grain are excellent candidate for marker assisted selection due to complex inheritance, high G × E interaction and difficult extraction protocols. From the results obtained in the present study it was apparent that, Y1ssr marker tightly linked with major QTL associated with individual as well as total carotenoids could effectively be used as an efficient gene targeted marker (GTM) for further

use in marker-assisted selection. The phytoene synthase (*psy*1) gene could be utilized for the development of functional marker (FM) for both pro-vitamin A as well as non pro-vitamin A carotenoids. The QTL detected on chromosome(s) 10 and 8 in this study, besides underlining the importance of these loci in regulation of isoprenoid pathway flux for carotenoids, can also help in unraveling the key enzymes of this pathway. In addition to QTL associated with candidate gene *psy*1 at *y1* locus, QTL cluster located at *y9* locus on chromosome 10 for control of the levels of carotenoids identified in the present study, may prove useful for pyramiding of favorable alleles to achieve higher levels of β-carotene, α-carotene, β-cryptoxanthin, zeaxanthin and total carotenoids from diverse germplasm. Finally, the results obtained in the present study are expected to lead to a better understanding of the genetic basis of carotenoids accumulation, and hence can assist in biofortification of maize with enhanced level of pro-vitamin A.

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