

QTL mapping for the tocopherols at milk stage of kernel development in sweet corn

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Abstract Tocopherols have several beneficial effects in plants, and are indispensable micronutrients for humans. Sweet corn is a major source of tocopherols in high concentrations. In this investigation, tocopherol compounds in sweet corn were analyzed by high performance liquid chromatography. To detect quantitative trait loci (QTL) controlling accumulation of tocopherols at the milk stage in sweet corn, a F₂ population consisting of 229 F_{2:3} lines was created from the cross between a high-total tocopherols line (A6) and a low-total tocopherols line (A57). A genetic map was constructed using 136 polymorphic molecular markers including one gene-targeted marker based on the tocopherol biosynthesis pathway (*HPPD*). Eleven putative QTLs for tocopherol content and composition were detected by composite interval mapping and located on Chr. 1, Chr. 2, Chr. 5, Chr. 6 and Chr. 10. Phenotypic variance explained by each QTL ranged from 4.74 to 41.16 %. Eight mapped QTLs were co-localized, suggesting that the same

QTL affected the amounts of more than one tocopherol compound. One candidate gene-targeted marker (*HPPD*) showed co-localization with the major QTL for γ -tocopherol and total tocopherols. Only one interval (umc1177–bnlg1429) on chromosome one exhibited a QTL for α , δ , γ , and total tocopherols with high LOD and R² values. The primary conclusion of this work is that two major QTLs located on Chr. 1 and Chr. 5 can be used for improvement of sweet corn nutrition quality by marker-assisted selection.

Keywords Sweet corn · Quantitative trait loci (QTL) · Tocopherols · Vitamin E

Introduction

Vitamin E describes eight natural compounds possessing α -tocopherol activity (Bramley et al. 2000; Eitenmiller 1997). The eight compounds are comprised of four tocopherols and four tocotrienols, which differ in the state of saturation of the side chain. Both tocopherols and tocotrienols include four sub-types: alpha (α), beta (β), delta (δ) and gamma (γ). These sub-types differ in the number and location of methyl groups. Vitamin E was first discovered in 1922 as a macronutrient essential to reproduction in rats (Brigelius-Flohe and Traber 1999). Since then, other important roles of vitamin E compounds in plants and animals have been demonstrated (Eggermont 2006; Sattler et al. 2003).

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Tocopherols are only produced in plants and have both photosynthetic and non-photosynthetic functions. In photosynthetic tissues, tocopherols are stored mainly in the chloroplasts to scavenge free radicals and to protect the photosynthetic apparatus from lipid peroxidation (Yamauchi and Matsushita 1979). In non-photosynthetic tissues, tocopherols are indispensable for the protection of polyunsaturated fatty acids (Goffman and Bohme 2001) and stored lipids from autoxidation. γ -Tocopherol is frequently the predominant form of vitamin E in plant seeds and in vegetable products.

Tocopherols are also indispensable for humans and animals, where they act as antioxidants to quench free radicals in cell membranes and protect polyunsaturated fatty acids from damage. Among the four tocopherols, α -tocopherol is the most important one for human health because it is highest in vitamin E activity (Kamal-Eldin and Appelqvist 1996). The lack of widespread vitamin E may cause an imbalance in tocopherol-regeneration systems and multiple inter-dependent antioxidant systems (Kuang Chow 2004). As a result, a surplus of free radicals may lead to oxidized products, capable of harming tissues. Tissue damage from free radicals is deemed to be related to chronic diseases such as cardiovascular disease, neurological disorders, cancer, cataracts, inflammatory diseases, and age-related macular degeneration (Bramley et al. 2000).

Natural α -tocopherol has been reported to have a higher biological activity than either other tocopherols or the synthetic form of α -tocopherol (Brigelius-Flohe and Traber 1999; Rocheford et al. 2002). α -Tocopherol has the highest known antioxidant activity in mammalian tissues, where one α -tocopherol molecule is effective at protecting 2,000 phospholipids (Bramley et al. 2000). One reason for the high level of α -tocopherol activity may be due to the manner of vitamin E absorption by the body. Vitamin E is absorbed with lipids in the intestine and transported to the liver. In the liver, α -tocopherol is specifically selected for absorption into the body by the hepatic α -tocopherol transfer protein. Because of the high efficiency of the transfer protein, α -tocopherol is ten times higher than γ -tocopherol in the plasma.

Although most research on vitamin E has focused on α -tocopherol, preliminary experimental studies suggest that γ -tocopherol in corn oil might possess a higher antioxidant capacity than α -tocopherol (Tomasch et al. 2001). One possible critical role of

γ -tocopherol is its effectiveness in quenching peroxynitrite, an electrophilic mutagen capable of damaging lipids, DNA and proteins (Brigelius-Flohe and Traber 1999; Rocheford et al. 2002).

Maize, a staple food for humans and animals, is the most widely grown grain crop in the world. Its kernels have been evaluated for tocopherol content, and γ -tocopherol has been found to be the most abundant (Grams et al. 1970). Even so, substantial natural variation of α , γ , and δ -tocopherols has been observed in corn kernels (Egesel et al. 2003; Grams et al. 1970; Rocheford et al. 2002). Thus, quantitative trait loci (QTL) analysis is a promising method for the study of tocopherols in maize. Furthermore, composite interval mapping (CIM) should increase the power of QTL detection and reduce bias in the estimated positions and effects of QTLs by combining interval mapping with multiple regression analysis to fully utilize the information in mapping data (Zeng 1994).

At the molecular level, reports concerning tocopherols in maize are scarce. Wong et al. (2003) mapped several QTL for tocopherols using an F_{2:4} mapping population and testcross progeny population. Chander et al. (2008) used a RIL segregation population to map sixteen genomic regions associated with tocopherols covering all the chromosomes except chromosome 4, and eleven genomic regions were colocalized with candidate genes. Therefore, rather than using expensive genetic engineering approaches to increase tocopherol concentration, breeding maize with high levels of tocopherols in the grain may provide an alternative source of vitamin E (Rocheford et al. 2002).

The tocopherol biosynthetic pathway in maize has not yet been well described, although it has been elucidated in *Synechocystis* and *Arabidopsis* (Mène-Saffrané and DellaPenna 2010). In maize, *VTE1* (a tocopherol cyclase) on chromosome 5 was the first gene in the pathway to be cloned, and it has been associated with the maize sucrose export defective1 (*SXD1*) locus (Provencher et al. 2001; Sattler et al. 2003). The second report of a tocopherol pathway gene concerned an α -tocopherol methyltransferase gene in which two insertion/deletions explains about 33 % of α -tocopherol variation.

Sweet corn is a type of maize with naturally occurring recessive mutations in genes controlling conversion of sugar to starch inside the endosperm of the corn kernels. Unlike field corn varieties, sweet corn is picked at the milk stage of kernel development

and is eaten by humans as a vegetable. Limited information about tocopherols in sweet corn is available. Thus, the objectives of this research were to examine the genetic inheritance of tocopherols in sweet corn kernels at the milk stage of development, along with relationships among tocopherols, and to detect QTL using an $F_{2:3}$ population.

Materials and methods

Plant materials and field evaluation

Sweet corn inbred lines A6 and A57 were selected from 47 inbred lines as the parents for this research on the basis of their extreme phenotypes for total tocopherols (data not shown). An F_1 was created from the cross of the two parents, and F_1 plants were self-pollinated to produce F_2 populations. F_2 plants were then self-pollinated to create a mapping population consisting of 229 $F_{2:3}$ families.

The 229 $F_{2:3}$ families and the two parents were grown at the South China Agriculture University experimental farm in a complete randomized block design with 3 replications in the autumn of 2011. Each family was planted with ten plants per row and management of the experimental field was the same as in production fields. All $F_{2:3}$ families and the two parents were self-pollinated and five plants of each $F_{2:3}$ family were harvested at the milk stage of kernel development. All ears were dried at 50 °C in a low-temperature drier and bulked at shelling in a dark room to avoid loss of tocopherol content in the kernels. For tocopherol analysis, approximately 35 g of randomly sampled kernels from five ears of each $F_{2:3}$ family were ground in a FW100 Stein mill for 2 min.

Tocopherol extraction and quantification

Tocopherol extraction and quantification by high performance liquid chromatography (HPLC) was performed according to Egesel et al. (2003). Tocopherols were separated on a reverse phase C_{30} column (5 μ m particle, 4.6 mm \times 25 cm), kept at 30 °C and analyzed by scanning from 200 to 800 nm, with a 2-nm bandwidth and a photodiode-array (PDA) detector connected to Shimadzu HPLC equipment. Detection at 295 nm and quantification of tocopherols was performed using external standards and six-point

standard curves. Tocopherol peaks in each sample were identified on the basis of co-chromatography, retention times, and by comparing absorption spectra on the photodiode array detector with those of authentic standards. Commercial standards for α -tocopherol, γ -tocopherol, and δ -tocopherol were purchased from Sigma (St Louis, MO, USA). Other chemicals (technical grade or higher) and chromatography solvents (HPLC-grade) used in this study were bought from Sigma or Merck (Darmstadt, Germany). The total tocopherol content of a sample was computed by summing the amounts of individual tocopherols (α , γ and δ -tocopherols). Tocopherol composition was expressed as the α -tocopherol/ γ -tocopherol ratio.

DNA extraction and linkage map construction

Leaf tissue was collected from seedlings of the parental and F_2 lines. Genomic DNA was extracted according to the CTAB method (Doyle and Doyle 1987) and preserved at -20 °C. Before running the polymerase chain reaction (PCR), DNA was diluted to a concentration of 25 ng/L with double distilled H_2O .

For molecular mapping, the two parents, A6 and A57, were screened with 512 SSR markers from the maize database (www.maizegdb.org). Six functional markers from candidate genes related to the tocopherol (*HPPD*, *VTE1*, *VTE3*, *VTE4*, *P3VTE5* and *P4VTE5*) were also included (Chander et al. 2008). Out of these 518 markers, 243 SSR markers and one functional marker for candidate gene *HPPD* were found to be polymorphic between the two parents and were, therefore, used to assay the 229 $F_{2:3}$ mapping populations. The forward primer sequence and the reverse primer sequence for candidate gene *HPPD* were 'GGACATGGGAGCTCGAATTA' and 'TTGC AAAGGAACAATTTTCAGG', respectively (Chander et al. 2008).

A linkage map with a minimum LOD score of 4.0 and a maximum distance of 40 cM was constructed using MAPMAKER/EXP V3.0 (Lincoln et al. 1992). Unlinked markers were assigned to their respective linkage groups using the 'try' command. Values of recombination fractions were converted into genetic map distances (cM) by means of the Kosambi mapping function (Kosambi 1944), and a sweet corn tocopherol linkage map was drawn using MapDraw2.1 (Liu and Meng 2003).

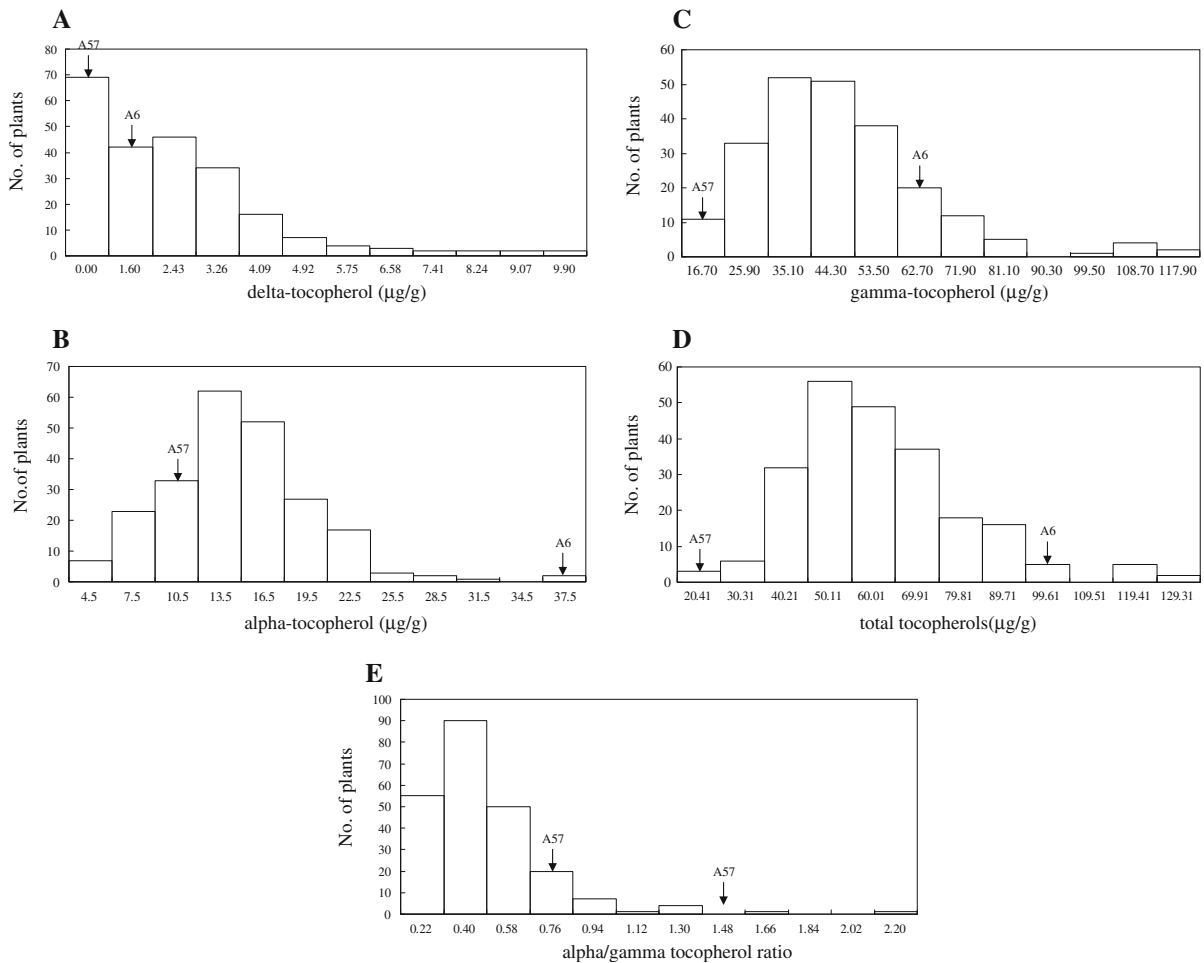


Fig. 1 Frequency distribution of tocopherol content and composition of the $F_{2.3}$ families population. Levels of the corresponding tocopherols in parents A57 and A6 indicated by

arrows, respectively. **a** Delta-tocopherol ($\mu\text{g/g}$), **b** alpha-tocopherol ($\mu\text{g/g}$), **c** gamma-tocopherol ($\mu\text{g/g}$), **d** total-tocopherol ($\mu\text{g/g}$), **e** alpha/gamma tocopherol ($\mu\text{g/g}$)

Data analysis and QTL mapping

QTL analysis was performed using Windows QTL Cartographer Version 2.5 (Wang et al. 2012). CIM was used to map the QTLs using percentage germination of each line averaged over three replications. An LOD value of 2.5 was used as the criteria to indicate the putative QTL position, with nomenclature following McCouch et al. (1997). The additive effect, dominance effect and the percentage of variance explained by individual QTLs were estimated. Gene action mode was calculated as described in Stuber et al. (1987). The Pearson correlation coefficients among traits were calculated using SAS V8.0 (SAS Institute, version 8.0, 1999).

Results

Phenotypic variation of tocopherol content and composition

Tocopherol content in A6 was greater than in A57 by more than 2-fold for α -tocopherol, seven fold for γ -tocopherol, and five fold for total tocopherol (Fig. 1). The δ -tocopherol content of A57 was zero, while A6 contained 1.6 $\mu\text{g/g}$, and a continuous distribution was observed in the $F_{2.3}$ families (Fig. 1). The ranges of α -tocopherol, δ -tocopherol, γ -tocopherol and total tocopherol content in the $F_{2.3}$ families were 1.67–35.3, 0–9.83, 7.79–117.12 and 10.51–128.75 $\mu\text{g/g}$, respectively. The range of α/γ -tocopherol ratio was

Table 1 Correlation coefficients among tocopherol content and composition, heritability estimates, and proportion of individual tocopherols in the F_{2:3} families population

Traits	δ-Tocopherol	γ-Tocopherol	Total tocopherol	α/γ-Tocopherol ratio	h ² B (%)	Amount of total tocopherols (μg/g)		
						Parents		F _{2:3} families
						A6	A57	
α-Tocopherol	-0.1660*	-0.0051 ^{NS}	0.2404**	0.6263**	0.83	36.11	10.87	13.39
δ-Tocopherol	1	0.3484**	0.3763**	-0.3737**	0.78	0.47	0.00	1.89
γ-Tocopherol		1	0.9655**	-0.5646**	0.89	57.59	7.63	41.35
Total tocopherol			1	-0.3991**	0.93	94.17	18.50	56.63
α/γ-Tocopherol ratio				1	0.76	0.71	1.42	0.39

^{NS} non significance, *h*²*B* heritability in broad sense

** and * significant at *p* = 0.01 and *p* = 0.05, respectively

0.04–2.13, which means the α-tocopherol content was higher than the γ-tocopherol content in some, but not all, F_{2:3} lines. The mean values of α-tocopherol, δ-tocopherol, γ-tocopherol, α/γ-tocopherol ratio and total tocopherol content in the F_{2:3} families were 13.39, 1.89, 41.35, 0.39 and 56.63 μg/g, respectively. Due to the extremely low amount of β-tocopherol found in maize, we did not measure β-tocopherol content in this study (Table 1).

The broad sense heritabilities for α-tocopherol, δ-tocopherol, γ-tocopherol, total tocopherols, and α/γ-tocopherol ratio were 83, 78, 89, 93 and 76 %, respectively (Table 1). Correlation coefficient analysis showed that a significant correlation of total tocopherols with individual tocopherols (Table 1), but with a closer relation to γ-tocopherol (*r* = 0.9655**) than to α-tocopherol (*r* = 0.2404**) and δ-tocopherol (*r* = 0.3763**). A significant negative correlation between α/γ-tocopherol ratio and all other tocopherol measures except for α-tocopherol was detected. The α-tocopherol content was positively correlated with total tocopherol and α/γ-tocopherol ratio (Table 1).

Construction of linkage map and QTL mapping

Of the 244 markers used to construct a linkage map, 136 were assigned to 10 chromosomes (Fig. 2). This linkage map had a total length of 2,031.2 cM with an average interval of 14.9 cM between adjacent markers. The marker order in this map is in good agreement with maize IBM maps (www.maizegdb.org).

A total of 11 putative QTLs for single tocopherol content and composition traits were detected on chromosomes 1, 2, 5, 6 and 10 based on CIM (Table 2;

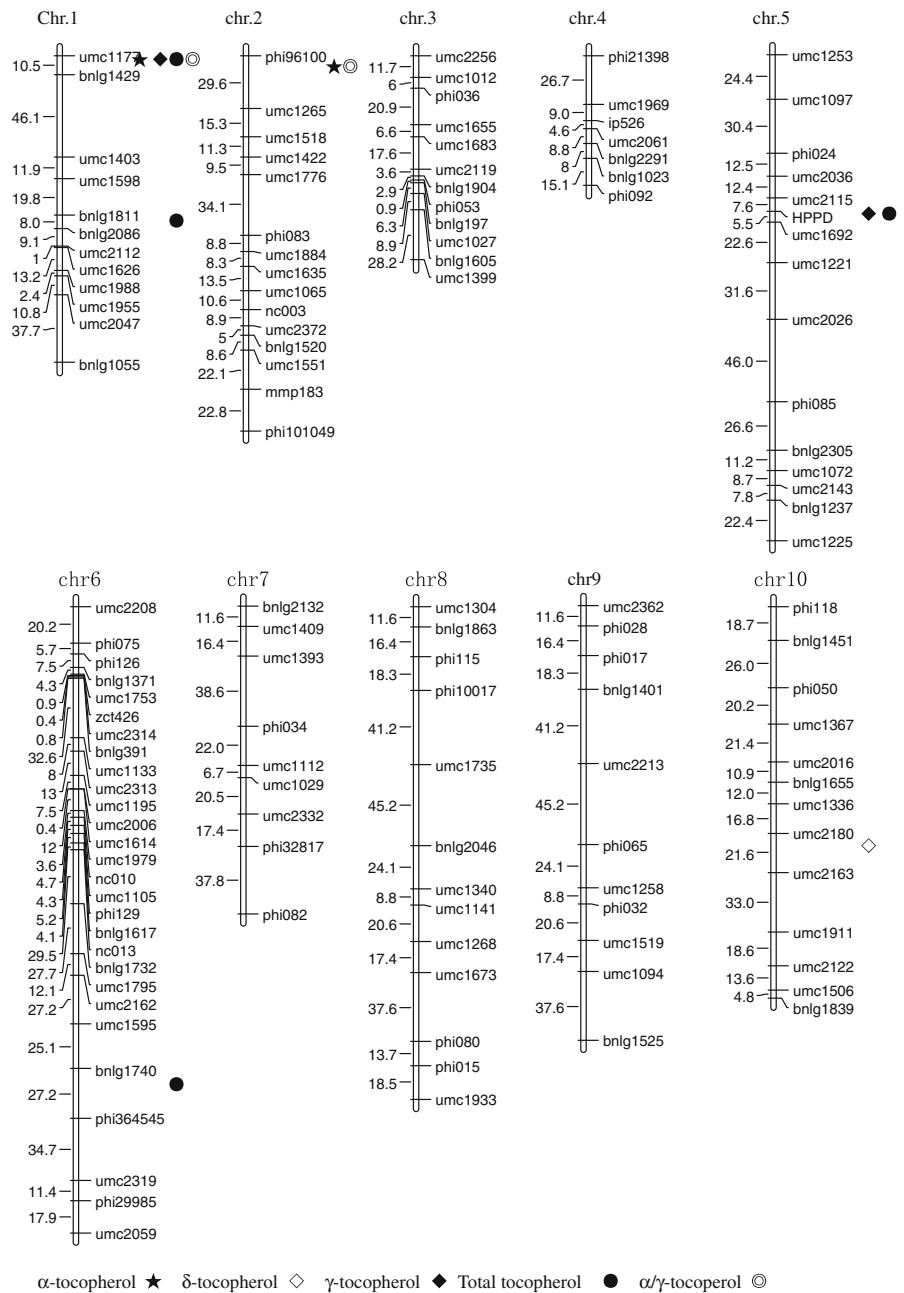
Fig. 2). Two QTLs accounting for 20.79 and 5.33 % of phenotypic variation were identified for α-tocopherol (Table 2). One QTL for δ-tocopherol accounted for 4.74 % of the phenotypic variation in the content of this compound in milk stage kernels. Two QTLs for γ-tocopherol accounted for 32.13 and 13.68 % of phenotypic variation for this trait. Four QTLs for total tocopherols accounted for 24.60, 6.18, 15.35 and 6.25 % of phenotypic variation. Two QTLs for total tocopherols were unique for this trait, while single QTLs also overlapped with QTLs for content of α-tocopherol or γ-tocopherol. The two QTLs for α-tocopherol also overlapped those for α/γ-tocopherol ratio and accounted for 41.16 and 5.83 % of variation in this ratio.

Among these chromosome regions, an interval located on chromosome 1 accounted for the highest proportion of phenotypic variation for all traits except for δ-tocopherol content. These major QTLs clustered in the umc1177–bnlg1429 interval and accounted for 20.79, 32.13, 24.60 and 41.16 % of the total phenotypic variation in α-tocopherol, γ-tocopherol, total tocopherols, and α/γ-tocopherol ratio, respectively. Parent A6 contributed all alleles favorable for increasing tocopherol content for the QTLs in this interval.

Overlapping QTLs with high LOD and explaining 13.68 and 15.35 % of γ-tocopherol and total tocopherol variation, respectively, were detected on chromosome 5. These two QTLs were located at position 67–112 cM with negative additive effects, suggesting that the favorable alleles at these loci were contributed by parent A6.

Chromosome 2 had one region in the phi96100–umc1265 interval associated with overlapping QTL,

Fig. 2 Genetic linkage map and locations of putative QTL for tocopherol content and composition detected in F_{2:3} families population derived from the cross of A6 × A57 (symbols indicate the positions of QTL for respective tocopherol content and composition)



one each for α-tocopherol and α/γ-tocopherol ratio. One minor QTL for α-tocopherol explained 5.33 % of the phenotypic variation and another minor QTL for α/γ-tocopherol ratio explained 5.83 % of the phenotypic variation. One QTL for total tocopherols

accounting for 6.25 % of total variation was detected in the bnlg1740–phi364545 interval on chromosome 6. These favorable alleles were contributed by A6. The only QTL detected for δ-tocopherol was located on chromosome 10 and explained only 4.74 % of the

Table 2 Putative QTLs for tocopherol content and composition in milk stage kernels of $F_{2:3}$ sweet corn families

Trait	Chr.	Flanking markers	Peak position (cM)	Range (cM)	LOD score	R ² (%)	Add
α -Tocopherol	1	umc1177–bnlg1429	1.01	0–2	4.69	20.79	–0.17
	2	phi96100–umc1265	0.01	0–4	2.81	5.33	–1.75
δ -Tocopherol	10	umc2180–umc2163	147.61	145–148	2.57	4.74	–0.34
γ -Tocopherol	1	umc1177–bnlg1429	2.01	0–7	11.37	32.13	–0.19
	5	umc2036–HPPD–umc1692	90.31	67–112	7.41	13.68	–10.20
Total tocopherols	1	umc1177–bnlg1429	2.01	1–6	6.54	24.60	–0.59
	1	bnlg1811–bnlg2086	94.31	92–99	2.69	6.18	1.56
	5	umc2036–HPPD–umc1692	90.31	67–113	8.57	15.35	–11.95
	6	bnlg1740–phi364545	290.01	272–301	3.07	6.25	–5.39
α/γ -Tocopherol ratio	1	umc1177–bnlg1429	1.01	0–1	12.44	41.16	–0.01
	2	phi96100–umc1265	0.01	0–7	3.05	5.83	–0.08

Chr. chromosome, LOD LOD score calculated by WinQTLCart 2.5, R² percentage of the phenotypic variance explained by each QTL, Add additive effect; positive and negative values indicate contributions from parents A57 and A6, respectively

phenotypic variation in content of this compound. The negative additive effect meant that the superior alleles were contributed by parent A6. Among all QTLs, parent A57 had one favorable allele explaining 6.18 % of phenotypic variation in total tocopherol located in the bnlg1811–bnlg2086 interval on chromosome 1.

In this study, a functionally characterized candidate gene HPPD involved in the maize tocopherol biosynthesis pathway was mapped in the $F_{2:3}$ population to the umc2036–HPPD–umc1692 interval on chromosome 5. QTL peaks for γ -tocopherol and total tocopherols were also mapped within a 4 cM region of the HPPD mapped location. The major QTLs covering HPPD explained 13.68 and 15.35 % of the phenotypic variation of γ -tocopherol and total tocopherol content, respectively.

Discussion

Human and animals acquire tocopherols mainly from plants. Sweet corn is widely consumed by humans, and its tocopherols have been studied by many researchers. In contrast to simple agronomic traits, selection for high vitamin E content is very expensive and labor intensive. Use of marker assisted selection (MAS) may be an efficient alternative to biochemical assays for selecting sweet corn with high vitamin E content.

The genotypes in this study contained 1.67–35.3 $\mu\text{g/g}$ α -tocopherol, 0–9.83 $\mu\text{g/g}$ δ -tocopherol, and 7.79–117.12 $\mu\text{g/g}$ γ -tocopherol. The main tocopherol was

γ -tocopherol, which comprised 73.02 % of the total tocopherol on average and is in agreement with previous work (Rochefford et al. 2002; Wong et al. 2003).

Significant positive correlations were observed between α and δ , and γ and δ -tocopherols. This indicates correlated responses of tocopherols. Total tocopherol content was positively associated with α , δ and γ -tocopherol, while α/γ -tocopherol ratio was negatively correlated with δ , γ and total tocopherols. These observations agreed with the results of Chander et al. (2008). Wide variation of tocopherol component contents, broad ranges of heritability estimates, and interrelationships among the components suggest enormous potential for biofortification of maize with an enhanced levels of tocopherols (vitamin E).

Assaying sweet corn tocopherol content by HPLC is expensive and labor intensive, particularly when a number of genotypes are being compared. QTL analysis has allowed geneticists and breeders to manipulate desirable traits via linked markers. In this study, eleven putative QTLs for tocopherols were detected, nine of which were clustered in three chromosome regions. This indicates the presence of clustered loci important for manipulating tocopherol content in a few key regions of the sweet corn genome.

In this study, overlapping QTLs located on chromosome 1 and flanked by umc1177 and bnlg1429 accounted for 20.79, 32.13 and 24.60 % of the α -tocopherol, γ -tocopherol and total tocopherol variation, respectively. A QTL in this region also

accounted for 41.16 % of the α/γ -tocopherol ratio variation. This appears to be a newly discovered region that is important in determining sweet corn tocopherol accumulation, and deserves more attention in future attempts to define and manipulate content of these compounds in this crop.

Another major QTL located on chromosome 5 and linked with marker phi085 contributed 33 % of phenotypic variation of α/γ -tocopherol ratio in a cross reported by Wong et al. (2003) In the current study, this region was associated with α -tocopherol, γ -tocopherol and total tocopherol. The combined results indicate that this chromosome region is an important determinant of α -tocopherol accumulation in sweet corn. Putative QTLs located on chromosome 5 explained 13.68 % of the variation for γ -tocopherol and 15.35 % of the variation for total tocopherols. This region flanked by umc2036 and umc1629 contains a candidate tocopherol-regulating gene, *HPPD*, which functions in converting *p*-hydroxyphenylpyruvic acid (HPP) to homogentisic acid (HGA). Gilliland et al. (2006) reported a major QTL explaining 40 % of the total phenotypic variation for α -tocopherol near the *HPPD* gene in *Arabidopsis*. In the results of Chander et al. (2008), this chromosome region explained 7.13, 7.13 and 9.31 % of γ -tocopherol, total tocopherol and α/γ -tocopherol variation, respectively. These results suggest that *HPPD* is a key determinant of tocopherol accumulation. Whether neighboring genes also have an effect remains to be determined.

A region of minor QTLs located on chromosome 2 flanked by phi96100 and umc1265 explained 5.33 % of the variation of α -tocopherol and 5.83 % of the variation of α/γ -tocopherol ratio. It was found to be linked with the marker umc1265 developed by Chander et al. (2008) as an indicator of tocopherol production. Another minor QTL associated with total tocopherol was located on chromosome 1 and explained 6.18 % of the total variation. This QTL flanked by markers bnlg1811 and bnlg2086, with bnlg1811 also found in the research of Chander et al. (2008). One minor QTL associated with δ -tocopherol and another minor QTL associated with total tocopherols were located on chromosomes 10 and 6, respectively. One major QTL and a few minor QTLs in this research were located near chromosome regions previously reported by Wong et al. (2003) and Chander et al. (2008). These results indicate that regulation of the tocopherol biosynthetic pathway in maize is similar to that in sweet corn.

It has been suggested that a few major-effect stable QTLs are typically desirable for MAS (Kulwal et al. 2005). In this research, two major QTL regions accounting for over 10 % phenotypic variation in associated tocopherol accumulation were detected on chromosome 1 and chromosome 5 near *HPPD*. This knowledge of key markers associated with regions responsible for tocopherol content and composition may be utilized in breeding programs for micro-nutrition biofortification of maize grain. It would facilitate the development of high micro-nutrition elite sweet corn varieties. This research indicates that the first step has been taken. With further work, high vitamin E sweet corn might be made widely available.

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