**Research Paper**

**Identification and epistasis analysis of quantitative trait loci for zeaxanthin concentration in maize kernel across different generations and environments**

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Zeaxanthin, a natural fat-soluble pigment, not only increases plant resistance, but also has vital significance for human health. However, quantitative trait loci (QTL) and the epistatic effects of zeaxanthin concentration in maize kernel have not been well studied. To identify QTLs and analyse the epistatic effects of zeaxanthin concentration in maize kernel, two sets of segregating generations derived from the cross between HuangC (a high zeaxanthin concentration inbred line) and Rezi1 (a low zeaxanthin concentration inbred line) were evaluated in three different environments. One major-effect QTL, qZea6a, explains 41.4–71.4% of the phenotypic variation and two QTLs, qZea4a and qZea3a, show LOD > 3 for zeaxanthin concentration detected over two generations and three different environments. Four of the ten QTL pairs show epistatic effects, explaining 7.34–14.3% of the phenotypic variance. Furthermore, additivity was the major allelic action at zeaxanthin concentration QTLs located in F₂ and F₂,3 populations and plants with homozygous HuangC alleles have a strong genetic ability in enhancing zeaxanthin concentration in maize kernel. These results will contribute to understanding these complex loci better and provide awareness about zeaxanthin concentration to maize breeders and scientists involved in maize research.

**Key Words:** linkage map, maize, quantitative trait loci, zeaxanthin.

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**Introduction**

Maize is one of the most important crops in the world, and is rich in protein, fat, starch, carotene, nicotinic acid, and vitamins B1, B2, and E. Orange maize is an essential staple cereal crop that naturally accumulates carotenoids in the edible seed endosperm, particularly zeaxanthin, accounting for about 0.1–9.0 mg/kg maize kernel (Sajilata et al. 2008). Zeaxanthin is a natural fat-soluble pigment. As an important part of the xanthophyll cycle, zeaxanthin has strong antioxidant, drought resistant, and high-salt tolerant abilities (Wu et al. 2015). Additionally, zeaxanthin can prevent the oxidation of lipids, vitamins and nutrients in foods, as well as maintain the flavours, and extend the shelf life of foods (Sajilata et al. 2008). Zeaxanthin is distributed in the eyes, kidneys, and liver of humans and animals. It plays an important role in preventing age-related maculopathy, and cataract, helps reduce the risk of cancer, and boosts immunity (Loane et al. 2008, Moeller et al. 2000, Nilsson et al. 2003, Snodderly 1995). However, since humans and animals cannot synthesise zeaxanthin de novo, they must obtain it through food (Sengun et al. 2014). Orange maize kernel has a high content of zeaxanthin while its concentration is extremely low in rice or green vegetables (Humphries and Khachik 2003, Perry et al. 2009). O’Hare et al. (2015) reported that the content of zeaxanthin in orange maize was significantly higher than that in yellow maize. Therefore, it is important to breed the commercial maize varieties with high zeaxanthin concentration, due to its benefit for human health.

Studies related to zeaxanthin have also been reported in *Arabidopsis*, tobacco and maize. β-carotene hydroxylase (*chyb*) is the key rate-limiting enzyme in the biosynthesis of zeaxanthin (Kato et al. 2004, Vallabhaneni et al. 2009). Davison et al. (2002) constitutively over-expressed the β-carotene hydroxylase gene in *Arabidopsis*, resulting in a 2-fold increase in zeaxanthin content in the lutein cycle. Moreover, overexpression of the β-carotene hydroxylase (*chyb*) gene significantly enhanced zeaxanthin in production tobacco, which dramatically increased the flux of the xanthophyll cycle (Wu et al. 2015). Vallabhaneni et al. (2009) showed that zeaxanthin content positively correlated with the amount of Hydroxylase3 (*HYD3*) transcript present, and also found that only *HYD3* was significantly associated with the β-carotene gene when compared to all carotene hydroxylase genes following analyses of their transcriptional levels. Yan et al. (2010) found that the gene *Zmcrtb1* was mainly associated with zeaxanthin in the...
maize endosperm.

To date, there have been only a few reports on QTLs of zeaxanthin in maize. Wong et al. (2004) identified some QTLs of zeaxanthin by using two sets of segregating generations—a set of $F_{2:3}$ lines derived from a cross between W64A and A632, and their testcross progeny with AE335. The results showed that three QTLs related to zeaxanthin were detected on chromosomes 6, 7, and 8, respectively and explained 6.5–19.8% of the phenotypic variation. Chander et al. (2008) selected 233 Recombinant Inbred Line (RIL) populations derived from the cross between By804 and B73 to construct genetic linkage maps, and the QTLs associated with zeaxanthin were identified on chromosomes 1, 6, 8, and 10, with 3.8–16.6% phenotypic contribution. These results showed many differences in QTL regions and contribution rates. Moreover, epistases between the QTLs were not analysed. Over-reliance on a single major QTL may lead to regardlessness important biosynthetic steps for which gene expression is controlled at interaction effect between different loci (Burt et al. 2011). Moreover, the possibility of combining epistatic effects with additive allelic effects from biparental populations for the creation of superior phenotypes in crops has been suggested (Latta et al. 2010). In the present study, four datasets from the $F_2$ and $F_{2:3}$ populations as well as three different environments were used. The objectives were (i) to detect the QTLs of zeaxanthin concentration in maize kernel and (ii) to analyse epistatic effects between the QTLs. We expect to identify the consistent QTLs for 16 h at 37°C and left undisturbed for 1 h. (3) The supernatant was centrifuged (6) The absorbance of the centrifuged liquid was determined at 445 nm using an Ultraviolet-Visible Spectrophotometer (Shanghai Tianmei Scientific Instrument). The entire process was conducted in a dark environment. The zeaxanthin concentration was determined using the following formula: \[
\text{Zeaxanthin (mg kg}^{-1}\text{)} = \frac{\text{OD}_{445} \times V}{W \times N \times f}; \text{OD}_{445} \text{ is the absorption value of the sample at the wave length of 445 nm, V represents the total volume of the sample used, W is the mass of the sample used, N is the dilution factor of the sample, and f is the specific extinction coefficient, which is 2.589 for zeaxanthin in n-hexane.}
\]

**Materials and Methods**

**Population construction**

Based on the results of our previous study (Deng et al. 2015), a high zeaxanthin concentration inbred line HuangC (1.91 mg*kg$^{-1}$) and a low zeaxanthin concentration inbred line Rezi1 (0.10 mg*kg$^{-1}$) were selected as parents for generating the cross (Fig. 1), HuangC × Rezi1. All the inbred lines were provided by the Maize Research Institute of Southwest University (China). The original cross, HuangC × Rezi1, was produced at Beibei (BB), Chongqing in the spring of 2015. Ten randomly selected $F_2$ and $F_{2:3}$ populations were constructed in a dark environment. The zeaxanthin concentration was determined using the following formula: \[
\text{Zeaxanthin (mg kg}^{-1}\text{)} = \frac{\text{OD}_{445} \times V}{W \times N \times f}; \text{OD}_{445} \text{ is the absorption value of the sample at the wave length of 445 nm, V represents the total volume of the sample used, W is the mass of the sample used, N is the dilution factor of the sample, and f is the specific extinction coefficient, which is 2.589 for zeaxanthin in n-hexane.}
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**Field experiment**

The $F_2$ generation and the two parental lines were planted at BB (29.45°N, 106.23°E, 249 m above sea level, loam soil) in the spring of 2016. The $F_{2:3}$ generations and the parental lines were planted at YJ (23.59°N, 102°E, 394 m above sea level, dry-red soil) in the autumn of 2016 while in BB and Hechuan (HC) (30.02°N, 106.15°E, 240 m above sea level, sand-loam soil), they were planted in the spring of 2017. There were a total of 286 plants in the $F_2$ generation and 242 families in the $F_{2:3}$ generation. The experimental design for the $F_{2:3}$ generation included a random complete block design in triplicates and 20 plants in each plot. All plants were self-pollinated to produce $F_{2:3}$ kernels.

**Zeaxanthin concentration determination**

Dry kernels from the $F_2$ and $F_{2:3}$ populations were ground into a powder for each ear and sieved with an 80-mesh sieve to obtain test samples. Concentration of zeaxanthin was determined using a spectrophotometric method with slight modifications (Deng et al. 2015, Xiuhong et al. 2013). Briefly, this included the following steps: (1) 5 mL of n-hexane: acetone (3:2, V/V) was added to a test tube containing 0.2000 ± 0.0001 g maize powder and was mixed well in the dark. (2) Samples were then incubated for 16 h at 37°C and left undisturbed for 1 h. (3) The supernatant was directly injected into a new tube, followed by the addition of 1 mL of 20% KOH-methanol solution for saponification reaction and then incubated for 1 h. (4) N-hexane (3 mL) and 10% sulphate solution (1 mL) were added to the tube for layering. (5) The supernatant was centrifuged (6) The absorbance of the centrifuged liquid was determined at 445 nm using an Ultraviolet-Visible Spectrophotometer (Shanghai Tianmei Scientific Instrument). The entire process was conducted in a dark environment. The zeaxanthin concentration was determined using the following formula:

\[
\text{Zeaxanthin (mg kg}^{-1}\text{)} = \frac{\text{OD}_{445} \times V}{W \times N \times f}; \text{OD}_{445} \text{ is the absorption value of the sample at the wave length of 445 nm, V represents the total volume of the sample used, W is the mass of the sample used, N is the dilution factor of the sample, and f is the specific extinction coefficient, which is 2.589 for zeaxanthin in n-hexane.}
\]

**Statistical analyses**

Significance tests of zeaxanthin concentration between
the parents were performed using Student’s t test. Variation analysis, skewness test and kurtosis test of zeaxanthin concentration distribution were conducted using SPSS Statistics 19.0 (IBM, 2010). Variances were estimated based on the general linear model: \( \chi^2_{ijk} = \mu + B_{jk} + G_{i} + E_{i} + (GE)_{ij} + e_{ijk} \), where \( B_{jk} \), \( G_{i} \), \( E_{i} \), \( (GE)_{ij} \), and \( e_{ijk} \) are the block effect (within the environment), genotype effect, environment effect, genotype and environment interaction effect and experimental error, respectively. All these effects are random. All calculations were performed using SPSS Statistics 19.0 (IBM, 2010) and Microsoft Office Excel 2016. The broad heritability of zeaxanthin concentration in F_{2:3} was calculated using the formula: \( h^2 = \sigma^2_G / (\sigma^2_G + \sigma^2_{GE} + \sigma^2_G/n + \sigma^2_e/nr) \), where \( \sigma^2_R \), \( \sigma^2_{GE} \), and \( \sigma^2_e \) are estimates of genotype variance, interaction variance between the genotype and the environment, and experimental error variance, respectively. \( n \) and \( r \) represents the number of environments and replicates, respectively (Knapp et al. 1985).

**Linkage map construction**

DNA was extracted from the leaves of F_{2} plants and the parental plants using the cetyltrimethyl ammonium bromide (CTAB) method (Causse et al. 1994, Murray and Thompson 1980). Simple sequence repeats (SSR) were used for molecular marker mapping (Senior et al. 1996). Amplified products were separated on a 6% polyacrylamide gel electrophoresis (PAGE) and visualized by silver-staining (Xu et al. 2002). Two hundred and forty-three polymorphism markers out of 1,331 SSR markers covering the entire maize genome (http://www.maizegdb.org) were applied to construct a linkage map using Joinmap version 4.1 (Ooijen 2011). The recombiant frequency between the linked loci was converted into genetic distance (centi-Morgan, cM) using Kosambi’s function (Kosambi 1943).

**QTL analysis**

The procedure of interval mapping was used to identify QTLs and estimate their effects on zeaxanthin concentration (Li et al. 2007, 2008, Wang 2009). QTL mapping was performed using the integrated software QTL 6.0 (https://www.kyazma.nl/). QTLs with similar marker intervals, identified from different environments for zeaxanthin concentration (overlapping-LOD confidence intervals provided by the software), were assumed to be the same. Parameters for forward regression analysis were set with a window size of 10 cM and a walk speed of 1 cM. Significance threshold for QTL detection was calculated by 1,000 random permutations of the phenotypic data at 5% level. Gene action mode \((d/a)\) of each QTL was estimated according to the ratio \((d/a)\) of the additive \((a)\) and the dominant \((d)\) effects. The data were classified as additive \((A; 0–0.20)\), partially dominant \((PD; 0.21–0.80)\), dominant \((D; 0.81–1.20)\), and over-dominant \((OD > 1.20)\) (Stuber et al. 1987, Tuberosa et al. 1998). The nomenclature of the QTLs were as follows: in \textit{gZea3a-F}_{2}, ‘q’ indicates QTL, ‘Ze’ is an abbreviation of the trait, namely zeaxanthin, the number ‘3’ represents the chromosome number, ‘a’ represents the order in which the QTL was detected, and ‘F_{2}’ indicates the F_{2} generation; for \textit{gZea4a-YJ}, ‘YJ’ represents the environment in which the QTL was identified. According to the constructed genetic linkage map and the analysed QTL results, Map Chart version 2.2 (Voorrips 2002) software was used to label the QTL linkage map.

**Allelic and epistatic effect analyses**

The segregation ratio among nine genotypes was examined using \( \chi^2 \) analysis \((a = 0.05)\). Multiple comparisons for zeaxanthin concentration in kernels among genotypes were conducted using SPSS Statistics 19.0 (IBM, 2010). QTL IciMapping, which was developed based on the Inclusive Composite Interval Mapping (ICIM) method and provided intuitive statistics for evaluating epistatic effects between the pairs of QTLs (Meng et al. 2015), was used to analyse the epistatic effects between the QTLs in the F_{2:3} population.

**Results**

**Phenotypic variation of zeaxanthin concentration**

The phenotypic results for zeaxanthin concentration are shown in Table 1. The two parents, HuangC and Rezi1, showed significant differences in zeaxanthin concentration among the two generations and the three different sites \((P < 0.01)\), which are indicative of the genetic differences between the two parents. A wide range of variation in zeaxanthin concentration is indicative of transgressive segregation and polygenic mode of inheritance.

**Heredity of zeaxanthin concentration in the F_{2:3} population**

From the analysis of genetic variance for zeaxanthin in the F_{2:3} population (Table 2), the values of \( \sigma^2_G \), \( \sigma^2_{GE} \), and \( \sigma^2_e \) were 0.1597, 0.2134, and 0.1018, respectively. The broad heritability of zeaxanthin was 65.96%, indicating that zeaxanthin was hereditable.

**Genetic linkage map of the F_{2} population**

A total of 243 SSR markers were used for constructing a genetic linkage map, and all the SSR loci were mapped onto ten linkage groups covering 2609.8 cM of the entire genome with an average interval of 10.73 cM between the adjacent markers (Fig. 2). The markers integrated on each chromosome were 18 to 31 pairs, and the mean value was 24.3. Most of the positions of the SSR markers were similar to the positions presented in the IBM 2008 Neighbours Frame 6 from Maize GDB. Therefore, the linkage map can be used for QTL analysis.

**QTL mapping in the F_{2} population**

The results of QTL analysis for zeaxanthin in the F_{2} population are shown in Fig. 2 and Table 3. A total of five QTLs were detected on chromosome 3, 4, 5, and 6. They...
Table 1. Phenotypic values of zeaxanthin content (mg*kg^-1 seed dry weight) of parents, F2 and F2:3 populations in three environments

| Population | Trait | Sites | P1          | P2          | Mean | Range   | Std error of mean | Skewness | Std error of skewness | Kurtosis | Std error of Kurtosis |
|------------|-------|-------|-------------|-------------|------|---------|------------------|----------|-----------------------|----------|-----------------------|
| F2         | BB    | YJ    | 2.1 ± 0.034** | 0.11 ± 0.025 | 1.06 | 0.02–3.23 | 0.04             | 0.38     | 0.15                  | -0.03    | 0.29                  |
| F2         | YJ    | BB    | 1.92 ± 0.031** | 0.37 ± 0.015 | 1.25 | 0.02–3.11 | 0.05             | 0.26     | 0.16                  | -0.78    | 0.32                  |
| F2         | BB    | YJ    | 1.92 ± 0.013** | 0.13 ± 0.013 | 0.92 | 0.02–3.36 | 0.04             | 0.77     | 0.16                  | 0.39     | 0.31                  |
| F2         | BB    | YJ    | 1.7 ± 0.023** | 0.13 ± 0.035 | 1.20 | 0.14–3.6  | 0.04             | 0.44     | 0.16                  | -0.25    | 0.31                  |
| F2         | BB    | YJ    | 1.7 ± 0.023** | 0.13 ± 0.035 | 1.20 | 0.14–3.6  | 0.04             | 0.44     | 0.16                  | -0.25    | 0.31                  |

** means significant at the 0.01 probability level.

Table 2. Variance components and heritability for zeaxanthin in the F2:3 population

| Items | Zeaxanthin |
|-------|------------|
| Genotype (σ_g^2) | 0.1597 |
| G × E (σ_ge^2) | 0.2134 |
| Error (σ_e^2) | 0.1018 |
| Heritability (h^2) | 65.96% |

σ_g^2, σ_ge^2 and σ_e^2 were estimates of genotype variance, interaction variance between genotype and environment, and experimental error variance, respectively.

explained 3.3–64.1% of the phenotypic variation and the LOD values of these QTLs were in the range of 2.07–62.75. qZea6a-F2, explaining 64.1% of the phenotypic variation, was located in the marker interval, y1–bnlg1422 (bin 6.01–6.02), on chromosome 6. On the chromosome 3, qZea3a-F2, explaining 61.1% of the phenotypic variation, was located in the interval bnlg1452–umc2263. In the interval umc2176–umc1963 on chromosome 4, qZea4a-F2 was detected with a LOD of 3.13. qZea5a-F2 (bin 5.07) on chromosome 5 accounted for 3.3% of the phenotypic variation for zeaxanthin concentration. Furthermore, the HuangC allele at these QTLs on chromosome 3, 5, and 6 increased zeaxanthin concentration, whereas the HuangC allele for QTLs on chromosome 4 decreased zeaxanthin concentration. The modes of gene action were over-dominance, additive and partial dominance, of which 60% of the QTLs favourable alleles originated from HuangC.

QTL analysis at different sites in the F2:3 population

In the F2:3 population, a total of 13 QTLs for zeaxanthin concentration were detected on chromosomes 1, 3, 4, and 6 at three different sites, of which three QTLs were major effect QTLs (Table 3, Fig. 2). qZea6a-YJ, qZea6a-BB, and qZea6a-HC were located in the common interval y1–bnlg1422 (bin 6.01–6.02), which explained 41.4–71.4% of the phenotypic variation, and LOD ranged between 26.72 and 65.76. The genes showed an additive effect and the favourable alleles of these QTLs originated from HuangC.

Other QTLs were located on chromosome 1, 3, and 4 (Table 3, Fig. 2), which explained 3.7–7.8% of the phenotypic variation. On chromosome 1, three QTLs (qZea1a-BB, qZea1a-HC, and qZea1b-HC), explaining 3.7–3.9% of the phenotypic variation, were detected at BB and HC sites. These genes also showed an additive effect and the favourable alleles of these QTLs originated from HuangC. On chromosome 3, four QTLs (qZea3a-BB, qZea3b-BB, qZea3c-BB, and qZea3a-HC), explaining 5.0–7.8% of the phenotypic variation, were detected at BB and HC sites and LOD values ranged between 2.68 and 3.68. On chromosome 4, three QTLs (qZea4a-YJ, qZea4a-HC, and qZea4b-HC), explaining 4.5–5.5% of the phenotypic variation, were detected at YJ and HC sites and these gene actions showed over-dominance. In addition, two QTLs (qZea3a-BB and qZea3a-HC) were located in the common region flanked by markers bnlg1452–umc2263 (bin 3.04) and explained 6.0–7.8% of the phenotypic variation. Moreover, the HuangC allele at all QTLs increased zeaxanthin concentration, except for qZea4b-HC.

QTL analysis for joint data of three sites in the F2:3 population

For joint data of the three sites in the F2:3 population, five QTLs for zeaxanthin were detected (Table 4, Fig. 2). These showed additive effects and the favourable alleles of the QTLs originated from HuangC. One of the QTLs, qZea6a-F2, located in the interval y1ssr–bnlg1422 on chromosome 6, was a major effect QTL, explaining 70.9% of the phenotypic variation. Four other QTLs (qZea1a-F2, qZea3a-F2, qZea4a-F2, and qZea7a-F2) explained 3.8–7.2% of the phenotypic variation and were located in the intervals of umc1833 and umc1147 on chromosome 1, bnlg1452 and umc2263 on chromosome 3, umc2176 and umc1963 on chromosome 4, and umc1407 on chromosome 7, respectively.

Allelic effect analysis of marker genotypes in the F2:3 population

In the F2:3 population, the number of individuals and the distribution of phenotypic values for these QTLs (qZea1a-F2, qZea3a-F2, qZea4a-F2, qZea6a-F2, and qZea7a-F2) at the nearest marker are shown in Fig. 3. The 242 plants were divided into three genotypes (homozygous HuangC, and heterozygous and homozygous Rezi1) based on their genotypes at five markers. For each of the five
markers, the genotype matched the phenotype. The zeaxanthin concentration for the homozygous HuangC genotype was 0.99–1.82 mg·kg$^{-1}$. Comparing among genotypes, a significant difference ($P < 0.01$) of zeaxanthin concentration between the two homozygous genotypes indicated that the y1ssr marker was related to zeaxanthin concentration and that the homozygous HuangC alleles had a strong genetic effect on improving zeaxanthin concentration in maize kernel (Fig. 3d).

Genotypic and phenotypic analyses at each combination of multiple marker genotypes (umc1833/umc1963, umc2263/umc1963, umc1963/y1ssr, and umc1407/y1ssr) are shown in Table 5. According to the law of independent assortment, a $\chi^2$ test showed that the nine genotypes tested in this study fitted the Mendelian separation ratio ($\chi^2 = 6.73–9.23, \chi^2_{0.05,8} = 15.51$). In addition, the zeaxanthin concentrations in kernels were highly significantly different among genotypes. When one allelic genotype was homozygous dominant (AA), the differences in zeaxanthin concentration between AABB and AABb, AABB and AAbb, and AABb and AAbb were not significant, highly significant and highly significant, respectively, for the umc1833/umc1963 and umc2263/umc1963 combinations, all highly significant for the umc1963/y1ssr combinations. When one allelic genotype was heterozygous dominant (Aa), the differences in zeaxanthin concentration between AaBB and AaBb, AaBB and Aabb, and AaBb and Aabb were not significant, highly significant and highly significant, respectively, for the umc1833/umc1963 and umc1963/y1ssr combinations.
discussion

In maize, zeaxanthin accumulates predominantly in the endosperm (Blessin et al. 1963, Steenbock and Coward 1925). Generally, yellow maize kernel contains higher amounts of zeaxanthin than white maize (O’Hare et al. 2015, Scott and Eldridge 2005). Quantitative traits, controlled by minor-effect polygenes, are highly susceptible to environmental effects (Flint and Mackay 2009, Holland 2007).

In previous studies, the QTLs for zeaxanthin concentration were mainly concentrated to the regions 6.01–6.02, 8.03–8.05, and 10.05–10.06 (Chander et al. 2008, Wong et al. 2004). Chander et al. (2008) identified a major effect QTL for zeaxanthin in the interval y1ssr–umc1595 on chromosome 6 (bin 6.01–6.02) by using a RIL.
population. Wong et al. (2004) detected the marker interval y1ssr–bnlg249 (bin 6.01–6.02) associated with qZea6a by using F2 population. In our study, the QTL qZea6a was located in the region of y1ssr–bnlg1422 (bin 6.01–6.02) within a distance of 1.2 cM in the F2 population. Comparing with the previous results, the region of y1ssr–bnlg249 associated with qZea6a is narrower than y1ssr–umc1595 on the IBM2008 neighbours Frame 6. This makes it possible to narrow down the QTL to a small genetic interval and segregate the QTL as a single Mendelian gene. Therefore, qZea6a shows a major effect on zeaxanthin concentration and stable expression across environments and genetic backgrounds, and is most important for marker-assisted selection (MAS) (Ribaut and Hoisington 1998).

It was previously reported that the QTL for zeaxanthin on chromosome 6 (bin 6.01–6.02) accounts for 13.9–16.3% of the phenotypic variation (Chander et al. 2008, Wong et al. 2004). In this study, the QTL qZea6a on chromosome 6 had a higher contribution to zeaxanthin production, ranging from 41.4% to 71.4%. This might be due to the high genetic difference in zeaxanthin genes between the two parents. Furthermore, the alleles producing higher increasing zeaxanthin concentration were the progeny of parents with high zeaxanthin production ability. Therefore, the parent with high zeaxanthin concentration is very important for the improvement of zeaxanthin in maize breeding programs.

To date, bin regions 6.01–6.02, 7.00–7.02, 8.03–8.05, 9.00–9.02, 10.00–10.02, and 11.00–11.02 have been identified as QTL regions for zeaxanthin production in maize. Among these regions, the region on chromosome 6 (bin 6.01–6.02) has the highest contribution to zeaxanthin production, ranging from 41.4% to 71.4%.

### Table 6. Epistasis analysis of QTLs across F2:3 population

| QTL × QTL | LOD (°) | R^2 (%) | Add by Dom | Dom by Add | Dom by Add |
|-----------|---------|---------|------------|------------|------------|
| qZea1a × qZea4a | 5.2 | 7.34 | 0.15 | -0.63 | -0.23 | -1.02 |
| qZea3a × qZea4a | 5.4 | 9.8 | 0.22 | 0.2 | 0.11 | 0.21 |
| qZea4a × qZea6a | 8.2 | 14.3 | 0.36 | -0.19 | -0.37 | -1.42 |
| qZea7a × qZea6a | 5.1 | 9.3 | 0.13 | 0.14 | -0.82 | -0.63 |

LOD: Log of odds; R^2 (%): Percentage of phenotypic variance explained by the QTL; Add by Dom: Additive by dominance effect at the two scanning positions; Dom by Add: Dominance by additive effect at the two scanning positions; Dom by Dom: Dominance by dominance effect at the two scanning positions.

### Table 5. Genotypic and phenotypic analyses at each of combinations of multiple marker genotypes in F2:3 population

| Maker genotype | Number of individuals | Mean of zeaxanthin concentration (mg*kg^-1) | Number of individuals | Mean of zeaxanthin concentration (mg*kg^-1) | Number of individuals | Mean of zeaxanthin concentration (mg*kg^-1) | Number of individuals | Mean of zeaxanthin concentration (mg*kg^-1) |
|----------------|-----------------------|--------------------------------------------|-----------------------|--------------------------------------------|-----------------------|--------------------------------------------|-----------------------|--------------------------------------------|
| qZea1a × qZea4a | umc1833/umc1963       |                                            | qZea3a × qZea4a       | umc2263/umc1963                          | qZea4a × qZea6a       | umc1963/y1ssr                          | qZea7a × qZea6a       | umc1407/y1ssr                          |
| AABB           | 12                    | 1.46a/A                                  | 13                    | 1.27b/B                                   | 13                    | 1.80a/A                                  | 13                    | 1.65b/B                                  |
| AABb           | 31                    | 1.40ab/A                                 | 39                    | 1.49a/A                                   | 31                    | 1.85a/A                                  | 37                    | 1.94a/A                                  |
| AAbb           | 12                    | 0.92f/E                                 | 14                    | 1.19c/C                                   | 13                    | 0.88de/D                                 | 14                    | 0.98d/D                                  |
| AaBB           | 29                    | 1.19cd/BC                                | 39                    | 1.29b/B                                   | 40                    | 1.84a/A                                  | 35                    | 1.20ce/CD                                |
| AaBb           | 55                    | 1.30bc/AB                                | 54                    | 1.27b/B                                   | 54                    | 1.52b/B                                  | 54                    | 1.24c/C                                  |
| Aabb           | 33                    | 1.00ef/DE                                | 29                    | 1.01d/D                                   | 27                    | 0.94cd/CD                                | 26                    | 1.00ed/CD                                |
| aaBB           | 23                    | 1.07de/CDE                               | 11                    | 1.05d/D                                   | 12                    | 1.03c/CD                                 | 12                    | 0.63/EF                                  |
| aaBb           | 34                    | 1.08de/CDE                               | 28                    | 1.00d/D                                   | 27                    | 0.80e/D                                  | 25                    | 0.59/F                                   |
| aabb           | 9                     | 0.62g/F                                 | 10                    | 0.70e/E                                   | 15                    | 0.38f/E                                  | 21                    | 0.43/F                                   |

^1 Lowercase letters indicate significance at the 0.05 level; Uppercase letters indicate significance at the 0.01 level.

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Fig. 3. The zeaxanthin concentration distributions and mean values for marker genotypes in F2:3 population. a–d: F2:3 population were genotyped at markers umc1833, umc2263, umc1963, y1ssr, and umc1407, respectively. The zeaxanthin concentration distributions and mean values are shown for homozygous HuangC, heterozygous, and homozygous Rezi1 genotypes. * means significant at the 0.05 probability level; ** means significant at the 0.01 probability level.

Breeding Science Preview
and 10.05–10.06 were reported to be associated with maize zeaxanthin concentration (Chander et al. 2008, Wong et al. 2004). In the present study, two other regions, bin 3.04 and bin 4.03–4.04, were detected to harbour QTLs for maize zeaxanthin concentration. Although these QTLs only contribute 5.0–7.8% to the phenotypic variation, they are worthy of further investigation since they are stable across different generations and environments. Several QTLs at different positions detected in different studies indicates that zeaxanthin concentration in maize is a complex quantitative trait.

The combination of epistatic effects and additive allelic effects from the two parents at different QTLs is related to the origin of transgressive segregation that produces superior phenotypes (Latta et al. 2010). In this study, the allelic effects of the QTLs varied among the F$_2$ and F$_{2:3}$ populations, and additive was the major allelic action at zeaxanthin concentration QTLs detected in the F$_2$ population, indicating that dominance was not the most important genetic basis for improving zeaxanthin concentration in the F$_{2:3}$ population. Moreover, epistasis causes hidden quantitative genetic variation in natural populations and could be responsible for the small additive effects (Mackay 2014). The LOD values and contribution rates ($R^2$) were different between a single QTL and a pair of epistatic QTLs. The four pairs of epistatic QTLs explained 7.34–14.3% of the phenotypic variance in the F$_{2:3}$ population, indicating that epistatic interactions for quantitative traits result in a change of magnitude of allelic effects at one locus. HuangC contributed to the favourable alleles involved in the epistatic interaction that increased the flux through the pathway.

Three of the twelve constitutive QTLs, $q$Zea3a, $q$Zea4a and $q$Zea6a, were observed to be constitutively expressed across the two generations and joint data of the three sites in the F$_{2:3}$ population. These QTLs may possibly perhaps have higher values of MAS for improving quality in maize. Furthermore, the closely linked molecular markers that we developed for $q$Zea6a will be useful in maize breeding programs for improving zeaxanthin concentration by applying MAS, and our results will also lay the foundation for the isolation of the candidate gene of maize zeaxanthin concentration.

**Author Contribution Statement**

EFD performed the fieldwork, collections of materials, data analysis, and manuscript drafting. YB and LPQ conducted the phenotypic measurement and DNA isolation. QYL and CXL were involved in SSR genotyping and PCR amplification. EFD and YLC was involved in experimental design and manuscript preparation. All authors read and approved the final manuscript.

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