Molecular Breeding (2005) 15: 257–269
DOI 10.1007/s11032-004-5947-8

Molecular genetic mapping of a high-lysine mutant gene (*opaque-16*) and the double recessive effect with *opaque-2* in maize

Wenpeng Yang[1,2,*], Yonglian Zheng[1,*], Wenting Zheng[1] and Rui Feng[1]
[1]*National Key Laboratory of Crop Genetic Improvement and National Center of Crop Molecular Breeding, Huazhong Agricultural University, Wuhan, Hubei 430070, P.R. China;* [2]*Institute of Upland Food Crops, Guizhou Academy of Agricultural Sciences and Guizhou Center of Maize Engineering Techniques, Guiyang, Guizhou 550006, P.R. China; *Author for correspondence (e-mails: ywpmaize@msn.com, zhyl@mail.hzau.edu.cn; phone: +86-27-87282689; fax: +86-27-87280016)*

Received 20 May 2004; accepted in revised form 8 November 2004

Key words: Gene pyramiding, High-lysine mutant, Maize, *opaque-16*, *opaque-2*, QTL mapping

Abstract

The lysin content in maize endosperm protein is considered to be one of the most important traits for determining the nutritional quality of food and feed. Improving the protein quality of the maize kernel depends principally on finding a mutant with a higher lysine content. Two high-lysine mutant lines with opaque endosperm, QCL3024 and QCL3021, were isolated from a self-cross population derived from Robertson's *Mutator* stocks. The gene controlling this mutation is temporarily termed *opaque-16* (*o16*). In order to illuminate the genetic locus and effect of the *o16* gene, two $F_{2:3}$ populations, one developed from a cross between QCL3024 and QCL3010 (a wild type line) and another from a cross between Qi205 (*opaque-2* line) and QCL3021, were created, and F_3 seeds from the F_2 plants in the two populations were evaluated for lysine content. The distributions of lysine content and tests for their normality indicate that the lysine content in the two populations is regulated by the major gene of *o16* and genes of *o2* and *o16*, respectively. Based on two data sets of the linkage maps of the F_2 plant marker genotypes and the lysine content of F_3 seeds originating from the two $F_{2:3}$ populations, the *o16* gene was located within 5 cM, at either 3 or 2.2 cM from umc1141 in the interval between umc1121 and umc1141 on the long arm of chromosome 8, depending on the recombination rate in the two populations as determined by composite interval mapping. According to the data of the $F_{2:3}$ population constructed from the *o2* and *o16* lines, the double recessive mutant effect was analyzed. The average lysine content of the F_3 '*o2o2o16o16*' families identified by the umc1066 and umc1141 markers was approximately 30% higher than that of the F_3 *o2o2* and '*o16o16*' families, respectively. The lysine content of seven F_3 families among nine F_3 double recessive mutant families showed different increments, with an average increase of some 6% compared with that of the maternal *o2* line. The potential application of the *o16* mutant for maize high-lysine breeding may be to combine it with the *o2* mutant bearing modifier genes, thus obtaining a mutant with much higher lysine content. For the purpose of pyramiding the *o16* with *o2* genes, the availability of closely linked markers of the *o16* and *o2* loci will facilitate marker-assisted selection and greatly reduce breeding time and effort.

Introduction

Storage proteins in cereal endosperm are deficient in some essential amino acids, especially lysine (Lys), tryptophan, and methionine. They constitute a large proportion of the total seed protein, causing the grain to be of inferior nutrient quality for monogastric animals. Protein supplementation

to correct such deficiencies is costly and wasteful of energy in animal nutrition, and is not feasible for most developing countries, which rely on cereal for human consumption. Therefore, increasing the amounts of protein-bound essential amino acids depends on improving cereal storage proteins.

Efforts to improve the protein quality of maize seed have focused on mutants with reduced zein synthesis and higher Lys content. A large number of mutants related to maize endosperm have been found, such as *o2*, *o6* (*pro1*), *o7*, *o15*, *Sh4* (*o9*), *o11*, *o13*, *fl1* (*o8*, *o4*), *fl2*, *fl3*, *De*-B30*, *Mc*, *Sh2*, and *bt2*, which are associated with Lys content and zein synthesis (Mains 1949; Teas and Teas 1953; Richardson 1955; McWhirter 1971; Soave et al. 1979, 1981; Nelson 1981; Salamini et al. 1983; Motto et al. 1989; Paulis et al. 1990; Dannenhoffer et al. 1995). The 'high-lysine' mutants first identified included *opaque-2* (*o2*) and *floury-2* (*fl2*) (Mertz et al. 1964; Nelson et al. 1965). The *o2* and *fl2* both reduce zein synthesis and increase synthesis of other endosperm proteins resulting in higher Lys content in their endosperm (Mertz 1992; Habben et al. 1993). To create high-lysine corn, enriched *o2* protein quality has been incorporated into agronomically important maize. More recently, modifier genes, or rather modified *o2* mutants, have been used in breeding programs for quality protein maize, i.e., high-lysine maize, to develop semi-vitreous and vitreous phenotypes with high Lys content and solve the problems associated with soft texture and pest susceptibility (Villegas et al. 1992; Cordova 2000; Shi et al. 2001). Along with these programs being put in practice, quality protein maize has been extensively used in developing countries.

Until now, efforts to seek more mutations with higher Lys content have been maintained in genetic and breeding programs for high-lysine maize. Segal et al. (2003) sought a dominant opaque endosperm mutation correlated with increased Lys content by RNAi, which could suppress the storage protein genes without interrupting O2 synthesis.

We isolated a higher lysine mutant with opaque endosperm from Robertson's *Mutator* (*Mu*) stocks, which contain mutations induced by insertion of *Mu* transposons (Robertson 1978). The gene controlling this high-lysine mutation was temporarily termed *opaque-16* (*o16*). To investigate the genetic basis of the variation of Lys content in this mutation, two $F_{2:3}$ populations were created from two sets of inbred lines. One population derived from inbred lines that differ in Lys content and endosperm phenotypes was used for molecular genetic mapping of Lys content, i.e., for mapping the *o16* gene. Another population with parental lines that contain the *o16* and *o2* gene, respectively, was utilized for remapping the *o16* gene and estimating the double recessive mutant effect. We report the locations of the *o16* gene in two molecular maps and the double recessive mutant effect of *o16* with *o2* gene. The information presented may be useful for marker-assisted selection and gene pyramiding in high-lysine maize breeding programs.

Materials and methods

Plant materials and population development

QCL3024 and QCL3021 are high-lysine mutant inbred lines with an opaque endosperm phenotype, and the Lys content of their kernels is higher than 0.36%. Both lines were isolated from a segregating ear 43D of a mutant line (5117) derived from Robertson's *Mu* stocks. QCL3010 is a wild type line with vitreous kernels, and the Lys content of its kernels is approximately 0.25%. QCL3010 was isolated from a segregating ear 46C of another mutant line (3142) also derived from the *Mu* stocks. To generate homozygotic state, these three lines were self-pollinated for five generations during 1994–1999. Qi205 is an *opaque-2* line with modifier genes (semi-vitreous endosperm phenotype), and the Lys content of its kernels is approximately 0.57%.

QCL3024 was used as the female parent in a cross with QCL3010. Two hundred and sixty-one F_2 plants were generated from an ear of the F_1 plants. The seeds of 164 F_3 ears were harvested from the F_2 plants. QCL3021, as the paternal parent, was crossed to Qi205. From F_1 of this single cross, 353 F_2 plants were produced. The seeds of 161 F_3 ears (families) were obtained from F_2 selfing plants. The F_2 plants from these two crosses were used for genotyping at SSR (simple sequence repeats) loci, and the F_3 seeds (families) harvested from each of the F_2 plants were utilized

for measuring the Lys content of the kernels. The measurements were considered representative of the phenotypes of the F_2 plants. The two sets of data were referred to as the two $F_{2:3}$ populations because the seeds of the ears on the F_2 plants were of the F_3 generation. The $F_{2:3}$ populations from the first and the second cross were named MF and PF populations, respectively.

All the field experiments were planted in the normal maize growing seasons. The S_1 to S_5 generations of QCL3021, QCL3024, and QCL3010 were planted at the Experimental Farm of Guizhou Academy of Agricultural Sciences, Guiyang; the F_1 to F_2 generations of the two crosses were grown in 2000 and 2001, respectively, at the Experimental Farm of Huazhong Agricultural University, Wuhan, China. All the seeds were thoroughly sun dried and stored at room temperature for 6 months before determination of Lys content in 2002.

Determination of lysine content

Chemical analysis of Lys content was performed by colorimetric methods using 2-chloro-3,5-dinitropyridine (adapted from Tsai et al. 1972; Zhao et al. 1982; Villegas et al. 1994; Wang 1998). Each of the F_3 seeds from the F_2 plants was classified according to kernel phenotype and mixed in proportion to its ratio. The mixed kernels were ground by CYCLOTEC® 1092 Sample mill (manufactured by Tecator, Hoeganaes), dried in a drying stove at 80 °C for 2 h, and defatted with ether for 8 h. One hundred milligrams of each sample was weighed and put into test tubes, and 5 ml papain solution (4 mg papain per ml phosphate buffer) was added to thoroughly wet the samples. A blank with just papain solution was included as a control. Tubes were incubated at 65 °C for 16 h (shaken at least twice in the first hour of incubation), then shaken, cooled to room temperature, and centrifuged at 2500 r/min for 5 min. The supernatant was transferred into centrifuge tubes in 1 ml aliquots, and then 0.5 ml carbonate buffer and 0.5 ml copper phosphate suspension was added. In the meantime, a stock solution of 2500 μg Lys per ml (62.5 mg Lys-monohydrochloride in 20 ml carbonate buffer) was diluted to 0, 250, 500, 750, 1000, and 1250 μg Lys per ml for a standard curve. To 1 ml of each of the solutions, 4 ml papain solution (5 mg papain per ml phosphate buffer) was added. One milliliter of each solution was transferred into centrifuge tubes and 0.5 ml amino acid mixture and 5 ml copper phosphate suspension were added. The mixtures of samples and the standard mixtures were shaken for 5 min and centrifuged at 2500 r/min for 5 min. The supernatant was transferred into large test tubes in 1 ml aliquots, 0.1 ml 2-chloro-3, 5-dinitropyridine was added, and the tubes were well shaken. The mixtures were allowed to stand at room temperature for 2 h with shaking every 30 min. Five milliliters of HCl (1.2 mol/l) was added to each test tube and shaken well. Five milliliters of ethyl acetate was added and mixed well by inverting the capped tubes at least 20 times. The upper phase was extracted three times, using a syringe adapted with a polyethylene tube (or a 5 ml pipetter with polyethylene tip). One milliliter of the aqueous phase was transferred to colorimetric cup, and the A value was measured with an ultraviolet spectrophotometer (DU® 520, manufactured by Beckman Coulter, California) at 390 nm after adjusting with the blank. The Lys content of samples was calculated using the following formula:

$$\mathrm{Lys}(\%) = \mathrm{D}/\mathrm{W} \times 10^{-3} \times 100,$$

where D is the weight (in μg) of Lys found from standard curve or computed by regression equation, and W is the weight (in mg) of sample.

Each of the F_3 seed samples was analyzed 2–4 times depending on difference between the two assays, and the mean of the Lys content for each of the F_3 seeds was calculated after eliminating dubious values by the Q-method (Wu 2002).

DNA preparation and SSR analysis

Genomic DNA from each of the F_2 plants derived from the two crosses and the parental lines was isolated from the young leaves following a procedure similar to that used by Saghai-Maroof et al. (1984).

Each amplification reaction contained 20 μl, consisting of 1×reaction buffer, 10% glycerol, 2 mmol of $MgCl_2$, 150 μmol of each dNTP mix, 0.3 μmol of each SSR primer, 0.75 U of Taq DNA polymerase, and 50 ng of genomic DNA. The reaction mixture was overlaid with one drop of

mineral oil. Amplifications were performed in a PTC-100 Programmable Thermal Controller (manufactured by MJ Research, Inc., Massachusetts) and T1 Thermocycler Modul 96 (manufactured by Biometro, Goettingen) programmed for the first denaturation step for 1 min at 93 °C, followed by 30 cycles of 1 min at 93 °C, 2 min at 58 °C, and 2 min at 72 °C, with a final extension for 5 min at 72 °C. The amplified fragments were separated onto 6% polyacrylamide sequencing gel containing 7 mol urea and visualized by the following silver staining procedure: the polyacrylamide gel was fixed twice in 10% ethanol + 0.5% glacial acetic acid for 6 min, or fixed once for 12 min, then rinsed with ddH_2O for 6 min, dipped in 0.2% $AgNO_3$ for 12 min of staining, rinsed with ddH_2O for about 12 s, and placed in 1.5% NaOH + 0.4% formaldehyde (37%) until DNA bands were displayed clearly. Later the gel was placed in 0.75% Na_2CO_3 for 3 min to end staining and finally rinsed with tap water for about 3 min and dried in air.

Linkage analysis and map construction

Genotyping of the F_2 individuals of the two populations was performed by SSR markers. A genetic map was constructed using the program MAPMAKER Version 3.0 (Whitehead Institute, Cambridge, MA; Lander et al. 1987; Lincoln et al. 1993). By means of the Kosambi mapping function (Kosambi 1944), the values of recombination fractions were converted into genetic map distance (cM). The map was drawn according to Liu and Meng (2003).

For the MF population, 341 SSR markers through the entire maize genome were screened between two parental lines of QCL3024 and QCL3010. Eighty-three markers with polymorphism (Table 1) were used to form the genetic map, of which 57 informative markers were assigned into 10 chromosomes of frameworks at a log of the odds ratio (LOD) threshold value of 8.0. The 57 SSR markers cover approximately 23% of the maize genome according to Bennetzen et al. (2001), averaging 12 cM for the marker intervals. The mapping and statistical analyses for Lys content were conducted based on the framework markers on this genetic linkage map (Figures 2 and 3).

In the PF population, 39 markers, selected around umc1066 and umc1141 on chromosome 7 and 8, respectively, were polymorphic between the parental lines of QCL3021 and Qi205. A linkage map of chromosome 7 and 8 was created. This map consisted of 22 framework markers assigned at an LOD threshold value of 8.2, with an average marker interval of 7.6 cM and total length of 151.0 cM (Figure 4). Based on the framework markers on this genetic map, the remapping of the mutation and statistical analyses were performed for Lys content of the PF population.

Table 1. Number of total markers and polymorphic markers on each chromosome examined between the parental line QCL3024 and QCL3010 of the MF population

Bin	Number of screened markers	Number of polymorphic markers
1.0–1.12	35	11
2.0–2.10	24	10
3.0–3.10	29	4
4.0–4.11	28	8
5.0–5.09	25	4
6.0–6.02, 6.04–6.08	30	6
7.0–7.06	26	9
8.0–8.09	91	24
9.0–9.08	35	5
10.0–10.07	22	8

Statistical analysis

Three methods, forward regression, backward regression, and forward and backward regression, of composite interval mapping (CIM) were used to examine the association between the Lys content rate and marker genotype with Windows QTL Cartographer Version 2.0 (North Carolina State University, Raleigh, NC) programmed by Wang et al. (2002). The parameters were set as follows: map function: Kosambi; distance units: centimorgan (cM); distance type: position; cross information: SF3 (self-cross F_3); walk speed: 2 cM; LR (likelihood ratio) threshold: 9.22 under H0:H3 (H0: $a = 0$, $d = 0$; H3: $a <> 0$, $d <> 0$), i.e., LOD = 2.0; CIM mode selection: model 6, i.e., standard model; background controls: 5 of control marker numbers and 10.0 cM of window size.

The normality of Lys level distribution was tested by Shapiro–Wilk Test using SAS software Version 6.0 (SAS Institute Inc., Cary, NC; Hui and Jiang 1996). The interaction effect between the

two markers was estimated by two-way ANOVA (analysis of variance; Liu 2003).

Results

Genetic analyses of lysine content in the MF and PF populations

The Lys content of the F_3 seeds from F_2 plants in the MF and PF populations was assayed by a chemical method. The distributions of Lys content of the two sets of F_3 seeds and the tests for the normality of the distributions were analyzed by SAS and Microsoft Excel 2000. The distributions of the two sets of Lys content data showed multiple peaks and transgressive segregation (Figure 1); tests for the normality of the two distributions showed that the Lys content in the two populations was not normally distributed ($p < W_{0.05}$). Hence, the phenotype of Lys content is controlled by one or two major genes.

In the MF population, the genotype of F_1 seeds harvested from the maternal plant are *O16o16* (*O16o16o16*), and the genotypes of the F_2 seeds harvested from an F_1 plant segregate in the proportion of 1 *O16O16* (*O16O16O16*):1 *O16o16* (*O16O16o16*):1 *O16o16* (*O16o16o16*):1 *o16o16* (*o16o16o16*). The genotypic constitutions of the F_3 seeds on the ears of the F_2 plants are as follows: 1/4 of the population is *O16O16* (*O16O16O16*), 1/4 is *o16o16* (*o16o16o16*), and individuals in the remaining 1/2 is a mixture of 1 *O16O16* (*O16O16O16*):1 *O16o16* (*O16O16o16*):1 *O16o16* (*O16o16o16*):1 *o16o16* (*o16o16o16*). Thus the segregation ratio of the population can be either 1:2:1 or 3:1, depending on the genetic effects of the alleles. By analogy with this reasoning, the segregation ratio at the *o16* and *o2* loci of the F_3 seeds in the PF population can be 9:3:3:1, 9:7, or 15:1, although it can be more complicated, also depending on the genetic effects of the alleles at each locus.

In the distribution of the Lys content of the MF population, a valley appeared to occur around 0.37%. Below and above this percentage, there were 122 F_2 plants or F_3 seeds and 42 F_2 plants or F_3 seeds, fitting a 3:1 segregation ratio ($p > 0.05$).

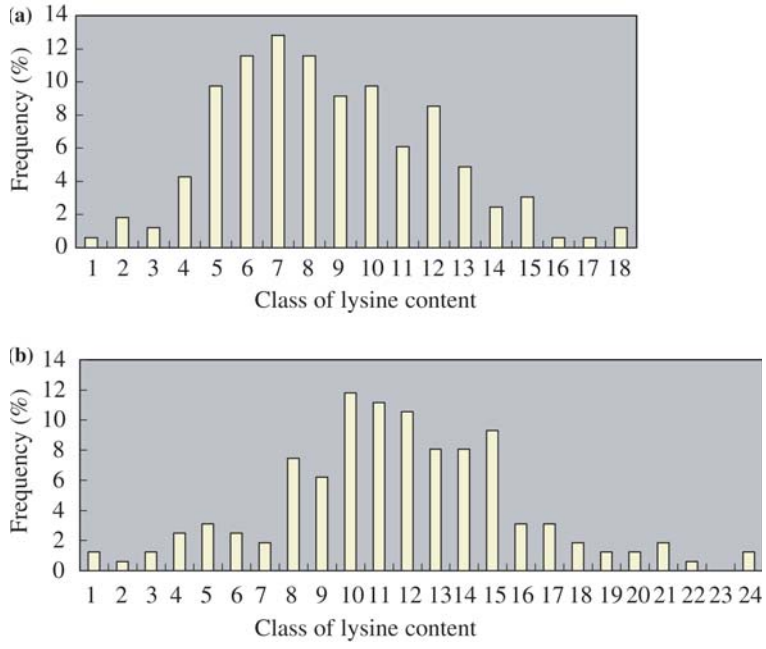

Figure 1. Frequency distribution (in percent) of Lys content of the F_3 seeds from F_2 plants in the MF and PF populations. (a) shows the distribution of Lys content of 164 F_3 seeds from F_2 plants in the MF population. (b) shows the distribution of Lys content of 161 F_3 seeds from F_2 plants in the PF population. The class numbers of Lys content in (a), from 1 to 18, stand for the 18 classes with a lower limit of 0.16%, uppermost limit of 0.52%, and class interval of 0.02%. The class numbers of Lys content in (b), from 1 to 24, stand for the 24 classes with a lower limit of 0.18%, uppermost limit of 0.66%, and class interval of 0.02%.

Thus, the segregation of the Lys content in this population was controlled by a single Mendelian locus, with the allele producing a higher Lys level showing a recessive effect. In addition, the continuous distribution of Lys content in the MF population seemed to indicate a dosage effect of the recessive allele, because genotypes with different numbers of recessive alleles would produce different Lys content. Similar to the above analyses, there seemed to be a valley from 0.42 to 0.46% in the distribution of the Lys content of the PF population. Below and above 0.42%, instead of 0.44%, there were 101 F_2 plants or F_3 seeds and 60 F_2 plants or F_3 seeds, fitting a 9:7 segregation ratio ($p > 0.05$). This indicates that the Lys content in the PF population was regulated principally by two single Mendelian factors and probably modified by the modifier genes of the maternal *o2* line.

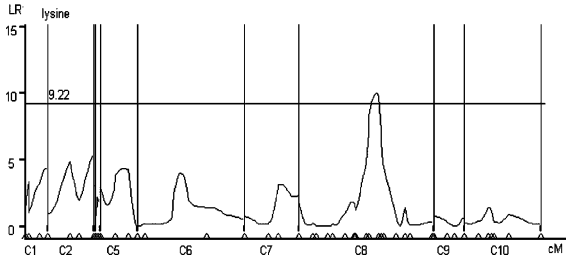

Figure 2. Results of composite interval mapping (CIM) using the forward regression method for the QTL associated with Lys content in the MF population. The plot is derived from CIM based on data from the $F_{2:3}$ population. The solid curves illustrate the likelihood ratio (LR) statistic. The LR scale is indicated on the left. The significant threshold of LRS is set to 9.22 (i.e., the LOD threshold value of 2.0, shown with horizontal line). C1–C10 denote chromosome 1 to chromosome 10, which are separated by vertical lines. The cM denotes genetic map distance converted by the Kosambi mapping function.

QTL mapping analysis for lysine content in the MF population

The Lys content in the MF population showed a quality-quantity trait, controlled by a major gene, with a phenotype of quantitative performance showing continuous variation. Therefore, quantitative trait locus (QTL) mapping analysis was performed for Lys content. To locate a gene, a QTL with a LOD peak value was defined as a position of a QTL, i.e., a locus of a major gene, and QTLs in the confidence region around a LOD peak value were ignored. The QTL with a LOD peak value, i.e., gene locus, was focused on in the following analysis.

To determine the genomic region for the *o16* gene controlling higher Lys content, CIM analysis based on the $F_{2:3}$ population of the MF population was conducted using the genotypes of 57 markers assigned on the linkage map of the maize genome. A QTL underlying Lys content, 3 cM from umc1141 in the interval between the umc1141 and umc1121 markers on chromosome 8, was identified by the Forward Regression method of CIM (CIMF) with a LOD score of 2.18 (Figures 2 and 3, Table 2). With the Forward and Backward Regression method of CIM (CIMFB), a QTL that was 5 cM from umc1141 was detected with a LOD score of 2.08. Both QTLs tested by the two methods could, respectively, explain more than 6% of the variance for the Lys content, and the primary effect was additive (Table 2). With the backward regression method of CIM (CIMB), although the peak value of the LOD score occurred 3 cM from the umc1141, the LOD score was below 2.0 and the QTL could not be considered to be identified, because the LOD value threshold 2 to 3 was suggested as significant for QTLs by Lander and Botstein (1989).

Because different QTL or chromosomal regions may need different window sizes (Wu et al. 1999), three window sizes (0, 10, and 20 cM on each side of the marker interval) were tried in order to identify the most appropriate one. The same results were obtained by CIMFM and CIMF. Therefore, the default window size (i.e., 10 cM on each side of the marker interval) was practical for the mapping of the QTL responsible for the Lys content in the present study.

Because each chromosome is genetically and statistically independent, QTL mapping can be conducted on a single chromosome to keep the desirable property of the interval test of CIM and achieve larger degrees of freedom for a small population (Wu et al. 1999). Hence, only markers on chromosome 8 were used to map the QTL underlying the Lys content, and a QTL that was 3 cM from umc1141 was identified by CIMF with a LOD score of 2.06 (Table 2 and Figure 3). The identified QTL could also explain over 6% of the variance for the Lys content and its primary effect was additive.

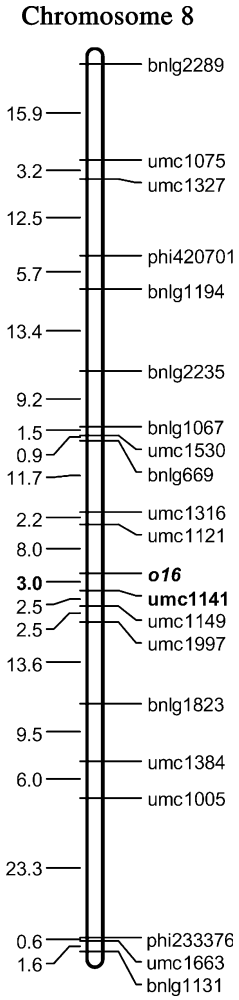

Figure 3. A local molecular linkage map of the F_2 population derived from a cross between QCL3024 and QCL3010 along with the position of *o16* by QTL analysis for Lys content in the $F_{2:3}$ population. The characters in boldface denoted the relative position of the *o16* gene.

This is consistent with the outcome achieved by employing CIMF with all genomic markers.

QTL remapping analysis for lysine content in the PF population

Using the PF population, remapping analysis for Lys content was conducted by CIMF, CIMB, and CIMFB. A QTL associated with variation in Lys content was positively identified on the long arm of chromosome 8 by these three methods. It was located at 2.20 cM from umc1141 in the interval between umc1141 and umc1121 and had a LOD score of more than 5.6 (Table 3 and Figure 4), which is even higher than the significance threshold ($LOD_{0.05}$ = 2.71) given by a permutation test repeated 1000 times on the two chromosomes at a significance level of 0.05. The result of the QTL mapping is actually identical with the result mapped with the MF population by CIMF. The 0.8 cM difference between the two results is the result of the difference between the genetic map distances in the MF and PF populations. The genetic map distance between umc1141 and umc1121 was 11 cM in the MF population; it was 10.2 cM in the PF population (Figures 3 and 4). Three window sizes (0, 10, and 20 cM) were also tried and the same results were obtained, indicating the QTL mapping results in this population are not influenced by window size.

Another QTL underlying Lys content at umc1066 on chromosome 7 was definitely detected by the three methods of CIM with a LOD score greater than 22.0 (Table 3 and Figure 4). It is just the locus of the *o2* gene, because umc1066 is a marker within the *o2* gene. In the meantime, the result showed the definition of the LOD peak value of a QTL as a gene locus to be reasonable.

The QTL on chromosome 8 (i.e., the *o16* locus) may contribute about 10% of the variance for the Lys content in the PF population. The QTL on chromosome 7 (i.e., the *o2* locus) may contribute

Table 2. Results of QTL mapping for Lys content in MF population

Method[a]	CIMF	CIMFB	CIMF[b]
Chrom	C8	C8	C8
Closest marker	umc1141	umc1141	umc1141
Pos. (cM)	3.00	5.00	3.00
LOD	2.18	2.08	2.06
A	−0.01	−0.01	−0.02
D	−0.03	−0.03	−0.03
H	6.46	6.69	6.71

[a]CIMF: composite interval mapping (forward regression method); CIMFB: composite interval mapping (forward and backward regression method); Chrom: chromosome; Closest: the closest marker from a QTL peak value; Pos.: the position (in centimorgan) of a QTL with the LOD peak value from the closest marker. LOD: peak value of LOD score. A: additive effect; D: dominant effect; H: heritability of QTL, i.e., proportion of phenotypic variance explained by QTL.
[b]Only the markers on chromosome 8 were used for mapping analyses.

Table 3. Results of QTL mapping for Lys content in the PF population[a]

Method	CIMF		CIMB		CIMFB	
Chrom	C7	C8	C7	C8	C7	C8
Closest marker	umc1066	umc1141	umc1066	umc1141	umc1066	umc1141
Pos. (cM)	0.00	2.20	0.00	2.20	0.00	2.20
LOD	22.61	5.87	22.66	5.63	22.11	7.22
A	0.08	−0.04	0.08	−0.04	0.08	−0.04
D	−0.03	−0.01	−0.04	−0.01	−0.04	−0.01
H (%)	39.81	9.56	39.67	9.10	40.03	12.45

[a]Abbreviations are the same as in Table 2.

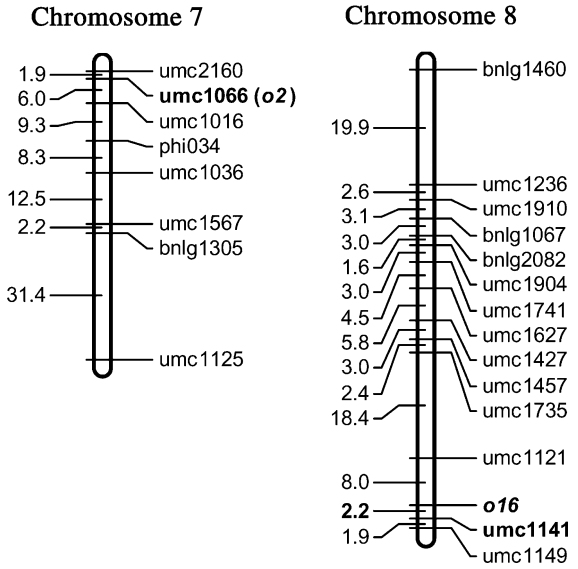

Figure 4. A molecular linkage map of chromosome 7 and 8 of another F_2 population derived from a cross between Qi205 and QCL3021. Boldface or boldface in parentheses denotes the relative positions of the mutant genes associated with the Lys content of F_3 seeds from F_2 plants.

some 40% of the variance for the Lys content, and its effect was estimated to be primarily additive rather than dominant (Table 3). The results showed a relatively higher contribution to the variation for the Lys content at the *o2* locus than the *o16* locus in PF population, possibly owing to the much higher Lys content of the *o2* line. Both QTLs (i.e., the *o2* and *o16* loci) could account for approximately 50% of the phenotypic variation in the Lys content, and they had very small additive and dominant effects, indicating the genetic effects on Lys content are complex, and may include other effects, such as maternal effect and dosage effect. Furthermore, Lys content is an endosperm trait, following a triploid inheritance model.

The effect on lysine content of the double recessive o16 *and* o2

In the PF population, the Lys content of nine F_3 double recessive '*o2o2, o16o16*' families, in which F_2 plants were detected by the umc1066 and umc1141 markers, was increased by approximately 30% on average, compared with the Lys content of the F_3 *o2o2* families. The Lys content of the F_3 '*o16o16*' families, in which F_2 plants were detected by the umc1141 marker, was on average equal to and the lower and upper limits of its range showed lower values than those of the F_3 *o2o2* families. In contrast, the Lys content of F_3 double dominant '*O2O2*, *O16O16*' families was decreased by an average of about 50%, compared with that of the F_3 *o2o2* families (Table 4). The results indicate that, compared with the wild type gene, a single high-lysine mutant gene could double Lys content; the pyramiding of two high-lysine mutant genes could nearly triple Lys content in maize kernel.

As compared with the Lys concentration of Qi205 (P_1), a parent with higher value, among nine F_3 double recessive mutant families identified by the umc1066 and umc1141 markers, the Lys concentration of seven F_3 families was approximately increased by a range of about 1–14%, with an average increase of 6%; but the Lys concentration of two F_3 families was decreased by 3 and 10%, respectively. Compared with the Lys concentration of QCL3021 (P_2), a parent with a lower value, the Lys concentration of all F_3 families from the F_2 double recessive mutant plants was increased by a range of 40–80%, with an average increase of 60% (Table 5). With regard to the lower increment of the Lys concentration of F_3 double recessive mutant families to P_1, the cause may be that the Lys content of the P_1 was much higher and even

Table 4. The Lys content of various genotypes of the double mutant of *o2* with *o16* in the PF population

Genotype A[a]	Genotype B[b]	Number of F3 families	Range of lys%[c]	Average of lys%	± % to '*o2o2, o16o16*'[d]
o2o2	'*O16O16*'	7	0.365–0.533	0.446	0.00
o2o2	'*O16o16*'	16	0.394–0.560	0.480	7.62
o2o2	'*o16o16*'	9	0.512–0.654	0.591	32.51
O2O2	'*O16O16*'	5	0.182–0.223	0.206	−53.81
O2O2	'*O16o16*'	22	0.258–0.473	0.347	−22.20
O2O2	'*o16o16*'	4	0.361–0.514	0.447	0.22
O2o2	'*O16O16*'	17	0.274–0.474	0.380	−14.80
O2o2	'*O16o16*'	50	0.254–0.493	0.379	−15.02
O2o2	'*o16o16*'	31	0.367–0.504	0.417	−6.50

[a]Genotype A denotes the genotypes at *o2* locus detected by umc1066.
[b]Genotype B denotes the genotypes at *o16* locus; the genotypes in quotation marks denote the genotypes are examined by umc1141.
[c]lys%, the percentage of Lys content in kernel.
[d]± % to ' *o2o2,O16O16*' indicates the percentage increase or decrease as compared with the average Lys% of ' *o2o2, O16O16*' genotype.

Table 5. The lysine content of the F_3 double recessive mutant families in the PF population

No. of the F_3 double recessive families	1	2	3	4	5	6	7	8	9
Lys%	0.512	0.555	0.579	0.582	0.586	0.598	0.609	0.643	0.654
± % to P_1[a]	−10.49	−2.97	1.22	1.75	2.45	4.55	6.47	12.41	14.34
± % to P_2	41.83	53.74	60.39	61.22	62.33	65.65	68.70	78.12	81.16

[a]P_1 was Qi205 with a Lys content of 0.572%; P_2 was QCL3021 with a Lys content of 0.361%. '± % to P_1 or P_2' indicates the percentage increase or decrease as compared with the Lys% of P_1 or P_2.

exceeded the upper limit of the Lys content of the F_3 *o2o2* families (Table 4). In addition, the increase or decrease of the Lys content of the F_3 double recessive mutant families compared with that of the parental lines may be related to the recombination between the two loci of the umc1141 and the *o16*. Furthermore, each of the F_3 double recessive mutant families possessed a different increment of Lys content compared with other genotypes. This suggests that the increase in the Lys content of corn endosperm was affected by its genetic background.

Table 4 shows that either the *o2* or *o16* gene has an additive effect on the Lys content, which is in accordance with the aforementioned results of QTL mapping. In addition, the interaction effect between the two loci of the umc1066 and the umc1141 was estimated with two-way ANOVA to examine the interaction effect between the *o16* and *o2* gene on the Lys content in the PF population. The estimative results were primarily additive × additive effects rather than other interaction effects. However, these effects were not significant ($p > 0.05$), which seems to indicate that the genetic effect of the *o16* and *o2* gene on the Lys content is independently accumulative.

Discussion

In the course of testing *Mu* populations for mutagenic activity, many opaque endosperm mutations have been found to segregate on self-cross ears. Some of these mutations are correlated with an elevated Lys content. The *o16* mutation, as temporarily named, is one of the examples isolated from the *Mu* populations. The *o16* mutants of QCL3021 and QCL3024 are associated with higher Lys content in their kernels. Because Lys content is generally considered to be the most important trait in determining the nutritional quality of maize, it is necessary to know the position of the *o16* locus on the maize genome and its genetic effect on Lys content. In this study, the results of the analysis of the MF population showed that the segregation for the trait of Lys content was controlled by a single locus. In other

words, the *o16* gene could regulate the Lys content in the MF population. Based on two sets of data, including the linkage maps and the Lys content of kernel samples derived from the MF and PF populations, the *o16* gene was located on the long arm of chromosome 8 through CIM, and the umc1141 was found to be linked to this gene within 5 cM. In addition, the double recessive mutant effect of the *o16* with *o2* gene was evaluated on Lys content, and the pyramiding of these two mutant genes could significantly increase the Lys content in maize kernels.

There are several reasons that we temporarily named the mutants isolated from *Mu* population as the *o16* mutants. First, some mutants with mutant genes on chromosome 8 are reported to have opaque and floury endosperm, such as the mutants with *dek20*, *dek29*, *fl**-*N1163*, *crp**-*N1429A*, *emp3*, *pro1* (*o6*), and *fl3* genes. The kernels of these mutants are nonviable, poorly viable, germless, or lethal at the seedling stage, except the *fl3fl3* mutant (Gavazzi et al. 1975; Nelson 1976, 1979; Neuffer 1985; Neuffer and Sheridan 1980; Sheridan and Neuffer 1980, 1982; Neuffer and England 1984; Sheridan et al. 1984, 1986; Manzocchi et al. 1985; England and Neuffer 1987; Neuffer and England 1994; Scanlon et al. 1994; Neuffer and England 1995; Neuffer et al. 1997). In contrast, the mutants of QCL3021 and QCL3024 grow normally and are able to produce seeds with opaque endosperm.

Second, *pro1* and *fl3* have already been localized in the 8.04 bin (http://www.maizegdb.org). Yang et al. (1995) and Yang (1998) screened out three RFLP markers linked with the *opaque-6* locus, bnl9.44, bnl9.08, and bnl7.08, using materials with different endosperm texture. In addition, bnl9.44, bnl9.08, and bnl7.08 reside in the 8.03, 8.03–8.04, and 8.04 bins, respectively (http://www.maizegdb.org). Ajmone-Marsan et al. (1992) reported that the recombination frequency between *O6* and *b32* is 4.0 ± 1.6%. The *b32* gene, which is also named as *rip1* (*ribosome-inactivation protein1*), was located in the 8.05 bin (http://www.maizegdb.org). However, two SSR markers of phi014 and umc1202 at the *rip1* locus were not polymorphic between parents of MF and PF populations. In our study, more markers in the 8.03, 8.04, and 8.05 bins than in other bins on chromosome 8 were examined for polymorphism between the parents of the MF population; and 4 of 36 in the 8.03 bin, 1 of 16 in the 8.04 bin, and 5 of 12 markers in the 8.05 bin were polymorphic. Only two marker loci among the polymorphic marker loci, the umc1121 and umc1141 loci, were associated with the Lys content by QTL analysis. In addition, the umc1121 and umc1141 loci are more than 50 cM farther from *rip1* according to a consensus genetic map (Polacco et al. 2003). Thus, the mutation responsible for QCL3021 and QCL3024 mutant lines is neither *o6* and *fl3* nor *rip1*.

Third, 389 kernels consisting of 284 vitreous and 105 opaque kernels were observed on the ear of the F_1 plant in the MF population. The segregation for the endosperm trait fits the ratio of 3:1 ($p > 0.05$). It indicates that the opaque endosperm mutation is recessive and related to higher Lys content. This is correlated with the result that the recessive allele is responsible for higher Lys content by the above analysis of distribution of Lys content in the MF population.

Fourth, the umc1066, phi057, and phi112 markers within the *O2* gene were used for examining the *O2* locus of the two lines. As shown in Figure 5, the band patterns of QCL3024, QCL3010, and QCL3021 were the same, but different from that of the *o2* line Qi205. This indicates that the viability of QCL3021 and QCL3024 is not related to the *o2* gene.

Molecular tagging of endosperm phenotype was used in an attempt to locate the *o16* and *o2* genes by means of MAPMAKER. All informative markers were not linked to the endosperm phenotype of F_2 kernels from the F_1 plant in the MF

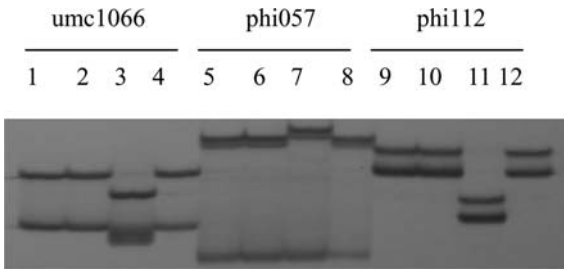

Figure 5. Detection of two mutant lines QCL3024 and QCL3021 at the *o2* locus. From left to right, lanes 1–4 were detected by umc1066, lanes 5–8 by phi057, lanes 9–12 by phi112; lanes 1, 5, and 9 were QCL3010, lanes 2, 6, and 10 were QCL3024, lanes 3, 7, and 11 were Qi205 (*o2*), and lanes 4, 8, and 12 were QCL3021.

population at an LOD threshold of 3.0. With an LOD threshold of 3.0 and without following the best order, the markers linked to the endospermous phenotype of F_2 kernels from the F_1 plant in the PF population would include umc1066 (5.2 cM) and umc1141 (17.5 cM). It is known that umc1066 is a marker within the *o2* gene, and the distance between the umc1066 locus and the *opaque-2* locus must be 0.0 cM (http://www.maizegdb.org). The 5.2 cM gap may be the result of endosperm heterofertilization. The genotypes of embryo and endosperm of heterofertilized kernels were different, and the heterofertilization rate was from 0 to 10% or even to 25% in different strains (Sprague 1929, 1932). This will result in the error of two-point and three-point tests in MAPMAKER when the molecular marker genotype of embryo (plant) and the phenotype of endosperm are used to find their linkage association. Another cause for the 5.2 cM gap may be the error of observation for the endospermous phenotype, because genic modification changes the endosperm phenotype of the *o2* mutants.

By molecular marker-based QTL analyses, Tan et al. (1999) revealed that three quality traits for the rice grain in regard to cooking and eating (amylase content, gel consistency, and gelatinization temperature) are controlled by a single locus that coincides with the *Wx* region on the short arm of chromosome 6. In our study, based on the Lys content of the F_3 seeds from F_2 plants in the PF population, the *o2* gene could be accurately positioned through QTL mapping. It is suggested that a Mendelian factor controlling a trait with continuous variation can be located by QTL mapping analysis. In this study, QTL mapping analyses were performed for the localization of a mutant gene (*o16*) controlling Lys content. In our case, although the markers nearly in each bin in the maize genome were examined (Table 1), the genetic map constructed with the MF population covers only a small portion of whole maize genome, probably due to the close genetic background of QCL3024 and QCL3010. It would be helpful in locating the major gene, namely the *o16* gene, through QTL mapping. The QTL identified in the MF population explains only 6% of the variability in the Lys content, as it may be due to environmental effects. It would be better if the F_3 population had been grown in multiple environments or years, but this would entail significant cost and effort. It would be better to grow another F_2 population in the same location within a year. With the PF population, the two QTLs account for approximately 50% of the variability in the Lys content. Although this is only a portion of the phenotypic variability, it is understandable compared with other QTL analyses. Based on an analysis of 176 mapping studies, QTLs commonly account for only about 50% of the phenotypic variability (Kearsey and Farquhar 1998). In addition, the occurrence of different LOD scores of the QTL (i.e., *o16*) between the MF and PF populations may be caused by differences in the growing environments of the F_2 plants, and the diverse LOD values and positions of the QTL among the three methods of CIMF, CIMB and CIMFB in MF population may be due to the algorithmic difference.

In addition, Lys content is an endosperm trait, and endosperm is triploid. Hence Lys content should follow the triploid genetic model. However, the statistical analyses of CIM are based on a diploid inheritance model. Wu et al. (2002a, b) have developed a maximum-likelihood-based method, implemented with the EM algorithm, to map QTL for endosperm traits, leading to an increase in the power of detecting a QTL affecting endosperm traits. However, the programs are being written in MATLAB of the University of Florida and there is not yet a publishable version for public use (personal communication, Wu R. and Ma C.X.), and they ignore the effect of the maternal genome on endosperm. It can be expected that the QTL and its effect on the endosperm trait will be revealed more accurately with the development of appropriate statistical methodology.

For the *o16* mutation, there is plenty of research still to be done, such as testing its allelism with the other endosperm mutants located on chromosome 8 to further verify whether it is novel, establishing near isogenic lines for studies of the molecular genetic basis of zein synthesis and Lys concentration in its endosperm, determining its combining ability with other elite inbred lines for yield, and evaluating its resistances to disease and pest to know its agronomic value, etc.

At present, the potential use for the *o16* mutation may be to incorporate it with the *o2* mutation bearing modifier genes, which has been improved for decades, to obtain a double recessive mutant with a better endosperm texture and an even higher

Lys content. In this case, our results can be valuable. In particular, the availability of closely linked markers of *o16* and *o2* loci will facilitate marker-assisted selection, and thus the two genes can be pyramided or transferred to other elite inbreds greatly reducing the breeding time and effort.

Acknowledgments

We greatly appreciate reviewers for their critical reviewing and constructive suggestions to our manuscript. The two segregating ears of 43D and 46C from *Mu* population were donated by Dr. J. L. Bennetzen, Purdue University, USA. The *opaque-2* line, Qi205, was offered by Dr. Shihuang Zhang, Chinese Academy of Agricultural Sciences. This research was supported by Grant (No. 39960040) from National Natural Science Foundation of China and visiting scholar Grant from the Educational Ministry of China to Wenpeng Yang; and by Hi-Tech Research and Development Program of China (Award No. 2001AA211111) to Yongliang Zheng.

References

Ajmone-Marsan P., Salamini F., Franceschini P., Monfredini G. and Motto M. 1992. The b-32 protein is not encoded by the *opaque-6* locus. Maize Genet. Coop. Newslett. 66: 20.

Bennetzen J.L., Chandler V.L. and Schnable P. 2001. National science foundation-sponsored workshop report. Maize genome sequencing project. Plant Physiol. 127: 1572–1578.

Cordova H. 2000. Quality protein maize: improve nutrition and livelihoods for the poor. CIMMYT Maize Res. Highl. 1999–2000: 27–31.

Dannenhoffer J.M., Bostwick D.E., Or E. and Larkins B.A. 1995. *opaque-15*, a maize mutation with properties of a defective *opaque-2* modifier. Proc. Natl. Acad. Sci. USA 92: 1931–1935.

England D.J. and Neuffer M.G. 1987. Chromosome 8 linkage studies. Maize Genet. Coop. Newslett. 61: 51.

Gavazzi G., Nava-Racchi M. and Tonelli C. 1975. A mutation causing proline requirement in Zea mays. Theor. Appl. Genet. 46: 339–345.

Habben J.E., Kirleis A.W. and Larkins B.A. 1993. The origin of lysine-containing proteins in *opaque-2* endosperm. Plant Mol. Biol. 23: 825–838.

Hui D.F. and Jiang C.J. 1996. A Practical Tutorial for SAS Software. Bei-Hang University Press, Beijing, pp. 1–74, (in Chinese).

Kearsey M.J. and Farquhar A.G. 1998. QTL analysis in plants: where are we now? Heredity 80: 137–142.

Kosambi D.D. 1944. The estimation of the map from the recombination values. Ann. Eugen. 12: 172–175.

Lander E.S. and Botstein D. 1989. Mapping Mendelian factors underlying quantitative traits using RFLP linkage maps. Genetics 121: 185–199.

Lander E.S., Green P., Abrahamson J., Barlow A., Daley M.J., Lincoln S.E. and Etoh T. 1987. MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1: 174–181.

Lincoln S.E., Daly M.J. and Lander E.S. 1993. Constructing Genetic Linkage Maps with MAPMAKER/EXP Version 3.0: A Tutorial and Reference Manual. Whitehead Institute for Biomedical Research Technical Report. 3rd edn. Whitehead Institute, Cambridge, MA, pp. 1–49.

Liu R.H. 2003. Dissection of Biomass Heterosis for Interspecific Oilseed Hybrid at Molecular Marker Level and Identification of Local Responsive Genes in *Brassica napus* for *Sclerotina*. Huazhong Agricultural University, Wuhan, China, PhD thesis, (in Chinese).

Lui R.H. and Meng J.L. 2003. Map draw: a Microsoft Excel macro for drawing genetic linkage maps based on given genetic linkage data. Hereditas (Beijing) 25: 317–321 (in Chinese).

Mains E.B. 1949. Heritable characters in maize. Linkage of a factor for shrunken endosperm with the *a1* factor for aleurone color. J. Hered. 40: 21–24.

Manzocchi L.A., Tonelli C., Gavazzi G., Di Fonzo N. and Soave C. 1986. Genetic relationship between *o6* and *pro-1* mutants in maize. Theor. Appl. Genet. 72: 778–781.

McWhirter K.S. 1971. A floury endosperm, high lysine locus on chromosome 10. Maize Genet. Coop. Newslett. 45: 184.

Mertz E.T. 1992. Discovery of high lysine high tryptophan cereals. In: Mert E.T. (ed.), Quality Protein Maize. American Association of Cereal Chemistry, St. Paul, MN, pp. 1–8.

Mertz E.T., Bates L.S. and Nelson O.E. 1964. Mutant gene that changes protein composition and increases lysine content of maize endosperm. Science 145: 279–280.

Motto M., Di Fonzo N., Hartings H., Maddaloni M., Salamini F., Soave C. and Thompson R.D. 1989. Regulatory genes affecting maize storage protein synthesis. Oxf. Surv. Plant Mol. Cell Biol. 6: 87–114.

Nelson O.E. Jr. 1976. The location of *fl3* on chromosome 8. Maize Genet. Coop. Newslett. 50: 114.

Nelson O.E. Jr. 1979. More precise linkage data on *fl3*. Maize Genet. Coop. Newslett. 53: 56.

Nelson O.E. Jr. 1981. The mutations *opaque-9* through *opaque-13*. Maize Genet. Coop. Newslett. 55: 68.

Nelson O.E., Mertz E.T. and Bates L.S. 1965. Second mutant gene affecting the amino acid pattern of maize endosperm proteins. Science 150: 1469–1470.

Neuffer M.G. 1985. Chromosome 8, short and long arms. Maize Genet. Coop. Newslett. 59: 109.

Neuffer M.G., Coe E.H. and Wessler S.R. 1997. Mutants of Maize. Cold Spring Harbor Laboratory, New York, pp. 1–468.

Neuffer M.G. and England D.J. 1984. Location of *Bif*, *Clt*-985* and *pro* on chromosome 8. Maize Genet. Coop. Newslett. 58: 77–78.

Neuffer M.G. and England D.J. 1994. *bif1-pro1-lg4* linkage on chromosome 8. Maize Genet. Coop. Newslett. 68: 27–28.

Neuffer M.G. and England D.J. 1995. Induced mutations with confirmed locations. Maize Genet. Coop. Newslett. 69: 43–46.

Neuffer M.G. and Sheridan W.F. 1980. Defective kernel mutants of maize. I. Genetic and lethality studies. Genetics 95: 929–944.

Paulis J., Bietz J.A., Bogyo T.P., Darrah L. and Zuber M.S. 1990. Expression of alcohol-soluble endosperm proteins in maize single and double mutants. Theor. Appl. Genet. 79: 314–320.

Polacco M., Sanchez-Villeda H., Coe E. and Columbia M.O. 2003. A consensus genetic map, Inter-mated B73 × Mo17 (IBM) Neighbors, 5718 Loci, July 2003. Maize Genet. Coop. Newslett. 77: 137–179.

Richardson D.L. 1955. Shrunken-floury, a gene affecting protein synthesis. Maize Genet. Coop. Newslett. 29: 45.

Robertson D.S. 1978. Characterization of a *mutator* system in maize. Mutat. Res. 51: 21–28.

Saghai-Maroof M.A., Soliman K.M., Jorgensen R.A. and Allard R.W. 1984. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc. Natl. Acad. Sci. USA 81: 8014–8018.

Salamini F., Di Fonzo N., Fornasari E., Gentinettta E., Reggiani R. and Soave C. 1983. Mucronate, *Mc*, a dominant gene of maize which interacts with *opaque-2* to suppress zein synthesis. Theor. Appl. Genet. 65: 123–128.

Scanlon M.J., Stinard P.S., James M.G., Myers A.M. and Robertson D.S. 1994. Genetic analysis of 63 mutations affecting maize kernel development isolated from mutator stocks. Genetics 136: 281–294.

Segal G., Song R. and Messing J. 2003. A new opaque variant of maize by a single dominant RNA-interference-inducing transgene. Genetics 165: 387–397.

Sheridan W.F., Chang M. and Neuffer M.G. 1984. The dek mutants – new mutants defective in kernel development. Maize Genet. Coop. Newslett. 58: 98–99.

Sheridan W.F., Clark J.K., Chang M. and Neuffer M.G. 1986. The dek mutants – new mutants defective in kernel development. Maize Genet. Coop. Newslett. 60: 64.

Sheridan W.F. and Neuffer M.G. 1980. Defective kernel mutants of maize. II. Morphological and embryo culture studies. Genetics 95: 945–960.

Sheridan W.F. and Neuffer M.G. 1982. Maize developmental mutants. J. Hered. 73: 318–329.

Shi D., Guo Q., Wang L., Meng S., Wen Y. and Guo Z. 2001. The situation of maize quality and development priority of high quality food maize in China. J. Maize Sci. 9: 3–7 (in Chinese).

Soave C., Tardani L., Di Fonzo N. and Salamini F. 1981. Zein level in maize endosperm depends on a protein under control of the *opaque-2* and *opaque-6* loci. Cell 27: 403–410.

Soave C., Viotti A., Di Fonzo N. and Salamini F. 1979. Recent evidence concerning the genetic regulation of zein synthesis. In: Leaver C.J. (ed.), Genome Organization and Expression in Plants. Plenum Press, New York, pp. 219–226.

Sprague G.F. 1929. Hetero-fertilization in maize. Science 69: 526–527.

Sprague G.F. 1932. The nature and extent of heterofertilization in maize. Genetics 17: 358–368.

Tan Y.F., Li J.X., Yu S.B., Xing Y.Z., Xu C.G. and Zhang Q. 1999. The three important traits for cooking and eating quality of rice grains are controlled by a single locus in an elite rice hybrid, Shanyou 63. Theor. Appl. Genet. 99: 642–648.

Teas H.J. and Teas A.N. 1953. Heritable characters in maize: description and linkage of brittle endosperm-2. J. Hered. 44: 156–158.

Tsai C.Y., Hansel L.W. and Nelson O.E. 1972. A colorimetric method of screening maize seeds for lysine content. Cereal Chem. 49: 572–579.

Villegas E., Ortega E. and Bauer R. 1994. Chemical Methods Used at CIMMYT for Determing Protein Quality in Cereal Grains. A Manual in Protein Quality Laboratory, International Maize and Wheat Improvement Center, Mexico City, pp. 17–20.

Villegas E., Vasal S.K. and Bjarnason M. 1992. Quality protein maize – what is it and how was it developed. In: Mertz E.T. (ed.), Quality Protein Maize. American Association of Cereal Chemistry, St. Paul, pp. 27–48.

Wang M.P. 1998. Analyses of Feed Nutrient Content. Huazhong Agricultural University Press, Wuhan, pp. 185–188 (in Chinese).

Wang S., Basten C.J. and Zeng Z.-B. 2002. Windows QTL Cartographer. WinQTLCart V2.0.

Wu M.C. 2002. Nutritional Ingredient Analysis and Sense Evaluation for Food. Chinese Agricultural Press, Beijing, pp. 17–27 (in Chinese).

Wu W.R., Li W.M., Tang D.Z., Lu H.R. and Worland A.J. 1999. Time-related mapping of quantitative trait loci underlying tiller number in rice. Genetics 151: 297–303.

Wu R., Lou X.Y., Ma C.X., Wang X., Larkins B.A. and Casella G. 2002a. An improved genetic model generates high-resolution mapping of QTL for protein quality in maize endosperm. Proc. Natl. Acad. Sci. USA 99: 11281–11286.

Wu R., Ma C.X., Gallo-Meagher M., Littell R.C. and Casella G. 2002b. Statistical methods for dissecting triploid endosperm traits using molecular markers: an autogamous model. Genetics 162: 875–892.

Yang W.P. 1998. RFLP markers linked with *opaque-6* locus of maize. Acta Agronom. Sin. 24: 34–41 (in Chinese).

Yang W.P., SanMiguel P., Stinard P., Robertson D. and Bennetzen J. 1995. Opaque mutations from *Mutator* self populations. Maize Genet. Coop. Newslett. 69: 135–136.

Zhao T.N., Chang Y.L. and Wang W.J. 1982. Analyses of Seed Quality (Chemical Composition). Scientific and Technical Press of Heilongjiang, Haerbin, pp. 76–81 (in Chinese).