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RESEARCH

Quantitative Trait Loci for Endosperm Modification and Amino Acid Contents in Quality Protein Maize

Andrés Gutiérrez-Rojas, Javier Betrán, M. Paul Scott, Halima Atta, and Mónica Menz*

ABSTRACT

The deficient protein quality of corn (*Zea mays* L.) grain can be improved by replacing normal *Opaque2* (*O2*) alleles with nonfunctional mutant *o2* alleles. Unfortunately, *o2* alleles are associated with soft endosperm texture, poor yield, and susceptibility to diseases and insects. Plant breeders have been able to restore a desirable ratio of hard to soft endosperm in *o2* germplasm. These modified genotypes are known as Quality Protein Maize (QPM). Neither the mechanism nor the genetic components controlling endosperm modification in QPM lines are well understood. Using a population of recombinant inbred lines, derived from a cross between an *o2* line and a QPM line, and a novel evaluation method for endosperm modification, quantitative trait loci (QTL) were mapped for traits related to the modification of endosperm texture and the content of the essential amino acids lysine, tryptophan, and methionine. Quantitative trait loci clusters for endosperm texture traits were detected on chromosomes 3, 5, 6, and 8, together accounting for 62 to 68% of the observed variation. For traits related to amino acid contents, QTL clusters were located on chromosomes 7 and 8, explaining up to 39% of the observed variation. The elucidation of the genetic mechanisms of the modification of *o2* endosperm and essential amino acid contents provides valuable information and important tools to plant breeders and plant scientists interested in improving the quality of cereal grains.

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Abbreviations: BLUP, best linear unbiased prediction; BSA, bulked segregant analysis; CIM, composite interval mapping; FAA, free amino acid; OPAC, opacity; PC, principal component; QPM, Quality Protein Maize; QTL, quantitative trait locus; RIL, recombinant inbred line; SSR, simple sequence repeat; TEXT, endosperm texture; VITR, vitreousness.

The endosperm of maize (*Zea mays* L.) kernel is a triploid tissue originating when a male gamete fertilizes the diploid central cell in a process parallel to the fertilization of the egg cell that gives origin to the diploid zygote. The main role of the endosperm is the synthesis and accumulation of storage products to nurture the embryo during initial stages of germination and seedling development (Costa et al., 2004). Reserves in the endosperm accumulate in the form of lipids, carbohydrates, and proteins. The structure and content of the endosperm affects traits targeted for genetic improvement such as grain yield (Vyn and Tollenaar, 1998), grain quality (Mazur et al., 1999), ethanol yield (Bothast and Schlicher, 2005), suitability for food processing (Paulsen and Hill, 1985), ruminal digestibility (Corona et al., 2006), and tolerance to mycotoxin accumulation (Bhatnagar et al., 2003).

Protein constitutes <10% of the kernel, while starch accounts for about 70%. In normal maize, 50 to 70% of the endosperm proteins are of the prolamin type (zeins). Zein proteins are often classified by

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differential solubility and mobility through gels as α -zein, β -zein, δ -zein, and γ -zein (Coleman et al., 1997). Zeins accumulate in subcellular compartments, derived from the endoplasmic reticulum, known as protein bodies (Lending and Larkins, 1989). The zein fraction is particularly deficient in the essential amino acids lysine and tryptophan. The high proportion of zeins in the endosperm, which results in a lack of lysine and tryptophan, is the primary reason for the poor protein quality of maize (Vasal, 2000).

Several genes affecting the composition and structure of the maize endosperm have been identified, of which *Opaque2* (*O2*) is one of the most extensively studied. Mertz et al. (1964) concluded that maize genotypes homozygous for the mutant allele *o2* had a considerably higher content of lysine and tryptophan in the grain when compared with wild-type genotypes. After this discovery, corn breeders started to introgress *o2* alleles into different germplasm, trying to improve nutritional quality, but undesirable traits were associated with this introgression. In particular, *o2* genotypes had soft kernels that were prone to mechanical damage, yield reductions of 8 to 15% relative to *O2* plants, and greater susceptibility to fungi and insect damage (Lambert et al., 1969).

The *O2* gene encodes a leucine-zipper class transcription factor (Schmidt et al., 1990) required for efficient transcription of a group of α -zeins. It also influences expression of other genes such as *b-32* (a Type I ribosome inactivating protein) and *CyPpdk1* (a cytosolic pyruvate orthophosphate dikinase) (Bass et al., 1992; Maddaloni et al., 1996). The soft endosperm texture of *o2* germplasm is associated with the reduction in the proportion of α -zein proteins (Huang et al., 2004). It has been postulated that the absence of specific zeins in *o2* genotypes causes the formation of smaller protein bodies and therefore alters the packing of the starch fraction during seed desiccation, resulting in abnormally soft endosperm (Schmidt et al., 1990). However, the endosperm of *o2* maize can be restored to resemble normal endosperm by the activity of modifier genes (Vasal, 1971). Modified *o2* genotypes with hard endosperm developed at the International Maize and Wheat Improvement Center (CIMMYT) are called Quality Protein Maize (QPM) (Vasal, 2000). In general, QPM genotypes retain higher levels of lysine and tryptophan than normal maize materials (Ortega and Bates, 1983).

Several studies have aimed to identify the genetic mechanisms responsible for the endosperm modification and grain quality in QPM maize. Lopes et al. (1995), using bulked segregant analysis (BSA) in populations developed from *o2* and modified varieties, found two loci associated with the modification of endosperm on chromosome 7. Holding et al. (2008) used BSA of two F_2 populations derived from QPM crossed to *o2*-detected markers linked to modifier loci on chromosomes 6, 7, and 9. In addition, a single-kernel and single-marker quantitative trait locus (QTL) analysis in one of these populations identified major QTLs on chromosomes 7 and 9 (Holding et al., 2008).

Based on these and other studies, it has been suggested that the γ -zein and its regulation are involved in the modification of *o2* endosperm (Burnett and Larkins, 1999). The 27-kDa γ -zein seems to be involved in the development of protein bodies and in the formation of a protein network that surrounds starch grains in vitreous endosperm (Dannenhoffer et al., 1995). In addition, differences in the branching pattern of starch and polypeptide levels of starch biosynthesis enzymes have been associated with modified phenotypes (Gibbon et al., 2003).

Quality Protein Maize genotypes have been introduced into production systems in several tropical and subtropical countries, where their nutritional advantages have been acknowledged. Nevertheless, there are major challenges to developing and using QPM germplasm, including the unknown number of modifier genes required to restore the desired hard-to-soft endosperm ratio, the need to evaluate grain quality during the breeding process, and genetic background effects (Belousov, 1987; Ciceri et al., 2000; Huang et al., 2004). Molecular marker-aided breeding has been used previously to convert normal lines into QPM lines (Babu et al., 2005); however, using markers for endosperm hardness and amino acid content along with *O2*-specific markers could significantly enhance the efficiency and reduce the cost of QPM breeding. Characterization of the genes involved in the modification of QPM genotypes will also provide valuable information for understanding the genetic basis of endosperm composition. The objective of this work was to identify QTLs for traits associated with the modification of endosperm in QPM maize, including endosperm texture and amino acid content. This study differed from all previous ones by using replicated data from a population of recombinant inbred lines (RILs) developed from the cross of an *o2* line with soft endosperm and a QPM line with harder endosperm evaluated in several locations and with a novel quantitative method, therefore increasing the precision and the power of the analysis.

MATERIALS AND METHODS

Plant Material

Details of the development of the population of RILs and the experimental design were reported by Gutierrez-Rojas et al. (2008). A population of RILs was derived from a cross between B73*o2* and CML161. CML161 is a tropical-lowland inbred classified as QPM and released by CIMMYT. B73*o2* is an *opaque2* conversion of B73, an Iowa Stiff Stalk inbred. A group of 146 RILs were used for field trials (S_5 , S_6 , and S_7 generations), and genotypic analysis (S_6 generation). The 146 RILs, the parental inbreds, and the reference inbred B73 were screened for the presence of a mutant allele *o2* using the *O2* gene-specific simple sequence repeat (SSR) markers phi057, phi112, and umc1066. Three RILs were removed from the analysis after showing unexpected fragment sizes for these markers. Additional sequencing and expression analysis corroborated the presence of a copy of the same *o2* allele in both B73*o2* and CML161 (Cruz-Vela et al., 2007).

Field Design

The population of RILs was evaluated in two Texas locations during 3 yr from 2004 to 2006, producing five environments: WE04 (Weslaco in 2004, one replication); WE05 (Weslaco in 2005, three replications); CS05 (College Station in 2005, two replications); CS06A (College Station in 2006 first planting, two replications); and CS06B (College Station in 2006 second planting, two replications). A randomized complete block design was used in all environments. Trials received common management practices according to each research station. At least 10 plants plot⁻¹ were self-pollinated. All plots were manually harvested.

Endosperm Texture Modification

The endosperm modification of the RILs was evaluated with three different measurements, endosperm texture (TEXT), opacity (OPAC), and vitreousness (VITR), which are associated with the extent of modification of the *o2* endosperm. TEXT was based on a visual rating from 1 (modified = flint-type round crown kernel and vitreous appearance) to 5 (opaque = dent-type kernels with very high proportion of floury endosperm) with increments of 0.5. A value of TEXT was assigned to self-pollinated ears that were harvested from each plot. OPAC was scored in a light box using a scale of 1 (modified = light passes through the whole kernel) to 5 (opaque = no light transmission due to completely opaque kernels) (Bjarnason and Vasal, 1992). An image analysis-based method was adapted to measure VITR (Leyva-Ovalle et al., 2002). Eight-bit black-and-white images were obtained by scanning longitudinally dissected kernels in a tabletop scanner (Hewlett-Packard ScanJet 3970, Palo Alto, CA). The negative of the image was used to estimate the area of soft (black) and hard (white) endosperm using the pixel counting option of the UTHSCSA Image Tool 3.0 software (Wilcox et al., 2002). VITR was defined as the percentage of the area of hard endosperm to the total endosperm area.

Amino Acid Composition

Tryptophan, methionine, and lysine were quantified using a microbiological method based on *E. coli* strains auxotrophic for tryptophan, methionine, or lysine as described by Scott et al. (2004). In this method, ground grain is extracted and proteins are enzymatically hydrolyzed in an acidic solution so both free and bound amino acids contribute to the measurement. Kernels from bulked ears from each plot were ground and measured in triplicate. The concentration of methionine, tryptophan, or lysine in each analysis was calculated using linear regression onto a standard curve developed using known amounts of pure amino acid standards.

Genotyping and Linkage Map

DNA was isolated from hypocotyl tissue obtained in bulk from 10 seedlings per RIL using a CTAB/sorbitol extraction buffer. Simple sequence repeat markers were selected from the Maize Genetics and Genomics Database (Lawrence et al., 2007) based on consensus map location. Simple sequence repeat markers were amplified by polymerase chain reaction and separated by electrophoresis in 4% (w/v) Super Fine Resolution agarose gels (Amresco, Solon, OH) using standard protocols. A linkage map was constructed in MapMaker/EXP 3.0b (Whitehead Inst. for Biomedical Research, Cambridge, MA) using a set of 180 SSR markers. The Kosambi's mapping function was used to transform recombination frequencies into map distances in centimorgans.

Statistical Analysis

Analysis of variance, phenotypic, and genetic correlations among the traits, and heritability estimates were reported by Gutierrez-Rojas et al. (2008). For the analysis of QTL, genotype least square means were estimated for each environment for each trait, and the best linear unbiased prediction (BLUP) procedure was used to predict the effects of each RIL across environments using univariate mixed-model analysis in PROC MIXED of SAS 9.1 (SAS Institute, 2003).

Principal Components Analysis

Principal components analysis was implemented separately for the endosperm texture traits and amino acid composition traits using the correlation coefficients in JMP version 7 (SAS Institute, 2007). Principal components (PCs) of correlated traits were used as quantitative traits in QTL mapping. Each PC is an uncorrelated linear combination of the original traits. The first PC (PC1) accounts for the greatest variance, and each of the subsequent PCs are uncorrelated combinations that explain less variation.

Quantitative Trait Loci Analysis

Analysis of QTLs was conducted with Windows QTL Cartographer version 2.5 (Bioinformatics Research Ctr., North Carolina State Univ., Raleigh, NC) for each trait using least square means calculated for each environment, BLUPs estimated across environments, or PCs of multiple traits. Composite interval mapping (CIM) was implemented using the forward and backward regression method (probability in = 0.1, probability out = 0.1), 5-cM window size, and 1-cM walk speed. Significance thresholds for the logarithm of the odds (LOD) scores corresponding to a Type I error rate of 5 and 10% were determined by permutation tests ($n = 1000$ permutations) (Churchill and Doerge, 1994). Significant QTLs detected by CIM were incorporated in multiple interval mapping (MIM) models to estimate their effects and to investigate possible QTL \times QTL interactions or epistasis. Multiple interval mapping allows simultaneous analysis of all putative QTLs and the inclusion of epistatic interactions.

RESULTS

The statistical analysis of the traits, phenotypic and genotypic correlations, and heritability estimates have been reported elsewhere (Gutierrez-Rojas et al., 2008). A summary table with mean phenotypic values and heritability estimates is shown in Table 1. The length of the linkage map constructed was 1798.1 cM, the mean marker interval distance was 10.05 cM, and 90% of the marker intervals were <20 cM (Fig. 1). The order of the loci in the linkage map was consistent with the consensus map of maize IBM2 2005 (Schaeffer et al., 2006).

Endosperm Texture Modification

Given the contrasting differences between the two parental inbreds, CML 161 (modified QPM endosperm) and B73o2 (opaque, non-QPM endosperm), the amount of variation observed was high for all measured traits. High heritability estimates (H entry-mean basis = 0.83–0.90) were calculated for the three measurements of endosperm modification

Table 1. Summary table for maize phenotypic values, heritability (Herit.) estimates, and standard error estimates for B73o2, CML161, and the recombinant inbred lines (RILs).

Trait [†]	Units	Parental inbreds		RILs						
		B73o2	CML161	Mean	SE	Range	Lowest value RIL	Highest value RIL	Herit. entry mean	SE (herit.)
TEXT	Score 1–5	4.66	1.74	3.23	0.06	1.87–4.72	RIL144	RIL63	0.9	0.014
OPAC	Score 1–5	4.92	1.75	3.59	0.07	1.84–4.87	RIL37	RIL63, RIL186	0.92	0.107
VITR	% hard/total area	9.97	46.54	29.45	0.80	9.51–51.42	RIL63	RIL92	0.83	0.030
Lys	Rel. [‡] units	0.10	0.09	0.09	0.00	0.08–0.11	RIL48	RIL89	0.75	0.034
Trp	Rel. units	0.20	0.14	0.17	0.00	0.14–0.20	RIL82, RIL282	RIL89	0.80	0.027
Met	Rel. units	0.13	0.10	0.11	0.00	0.10–0.13	RIL5	RIL221	0.71	0.040

[†]TEXT, texture; OPAC, opacity; VITR, vitreousness; Lys, lysine; Trp, tryptophan; Met, methionine.

[‡]Rel., relative.

(Gutierrez-Rojas et al., 2008). Quantitative trait loci for the three traits on chromosomes 1, 3, 4, 5, 6, 8, and 10 were detected with CIM analysis (Fig. 1 and Table 2). For TEXT, nine QTLs showed significant LOD scores across all environments. These QTLs together explained 76.9% of the phenotypic variation (Fig. 1 and Table 2). For the QTLs in bins 3.02 (phi374119), 3.05 (umc1539), 3.07 (Txp196L), 5.05 (umc2026), 8.05 (umc1562), and 10.06 (umc1061), the allele increasing modification was from the QPM parent. For QTLs in bins 1.06 (umc1035) and 6.06 (umc2170), the allele that decreased the proportion of opaque endosperm derived from B73o2. The QTL in bin 6.06 near to the marker umc2170 explained 23.1% of the phenotypic variation, and the QTL in bin 8.05 near to umc1562 explained 19.5%.

The trait OPAC was based on the observation that opaque kernels transmitted less light than the modified kernels. The CIM analysis detected six significant QTLs for OPAC (Fig. 1 and Table 2). These QTLs explained 62.5% of the observed variation. The alleles that decreased opacity in the QTLs in bins 3.02 (phi374119), 3.05 (umc1539), 3.06 (phi102228), 5.05 (umc2111), and 8.05 (bnlg1599), were derived from the QPM parent and the allele that decreased the proportion of opaque endosperm at the QTL in bin 6.06 (umc1912) was derived from B73o2. The QTL explaining a higher percentage of variation was mapped in bin 8.05 (bnlg1599), accounting for 17.3% of the variation. The interaction that explained the largest amount of observed variation was between the QTLs in bins 1.00 and 6.06 with 3.8%.

The VITR was measured through analysis of digital images of dissected endosperms, a process that allowed a quantitative measure of the areas corresponding to soft and hard endosperm in representative kernels of each RIL. The CIM analysis detected seven significant QTLs for VITR (Fig. 1 and Table 2). These QTLs explained 71.9% of the observed variation. For the QTLs in bins 3.02 (phi374121), 3.05 (umc1539), 4.06 (umc2027), 5.05 (bnlg1237), and 8.05 (bnlg1599), the alleles that increased the proportion of hard endosperm were derived from the QPM parent. The alleles that increased the proportion of hard endosperm in QTLs in bins 1.11 (phi064) and 6.06 (umc2170) were derived from the non-QPM parent. The main QTL for this trait was in bin 6.06 (umc2170), explaining 27.5% of the observed variation.

The first two PCs accounted for 95% (PC1 = 87.8% and PC2 = 7.2%) of the variation in the three endosperm texture traits. Quantitative trait locus mapping of PC1 identified five major QTLs (Fig. 1 and Table 2). These five QTLs correspond to QTL clusters, or “hot spot,” regions where CIM analysis detected QTLs for all three traits. There was a QTL cluster on chromosome 3S (bins 3.03–3.04); on chromosome 3L (bins 3.05–3.08); on chromosome 5L (bins 5.04–5.05); on chromosome 6L (bins 6.06–6.07), with one well-defined QTL peak for which the allele that contributes to texture modification came consistently from the non-QPM parent B73o2; and on chromosome 8L (bins 8.05–8.06).

Amino Acid Content

The parental lines showed significant differences for the relative content of the essential amino acids lysine, tryptophan, and methionine. B73o2 showed higher levels than the QPM parent for all three amino acids (Gutierrez-Rojas et al., 2008). Across environments there were three significant QTLs for lysine, representing 32.9% of the observed variation (Fig. 1 and Table 3). The main QTL was found in bin 8.03 near the marker bnlg1863, explained 14.8% of the variation, had the favorable allele derived from the non-QPM parent. Interestingly, the alleles that increased the content of lysine in the QTLs in bins 1.06 (umc1035) and 5.06 (phi087) were from the QPM parent. The percentage of the observed variation explained by these QTLs was 12 and 6.1%, respectively.

Six QTLs were identified for tryptophan content (Fig. 1 and Table 3), that explained 49.1% of the observed variation. The alleles that increased tryptophan content of QTLs in bins 6.04 (umc1014), 7.02 (umc2142), 8.02 (umc1304), 8.04 (umc1460), and 10.04 (umc1678) were from the non-QPM parent. For the QTL in bin 5.07 (bnlg118), the allele that increased the content of tryptophan was derived from the QPM parent. The more significant QTL was the one in bin 10.04 (umc1678) explaining 16.4% of the variation. The interaction between the QTLs in bins 3.03 and 5.06 explained 5.6% of the observed variation.

Four QTLs were identified for methionine content (Fig. 1 and Table 3). These QTLs together explained 57.3% of the observed variation. In all the QTLs (bins 2.08, 5.03, 7.02, and 8.02), the favorable alleles that increased the

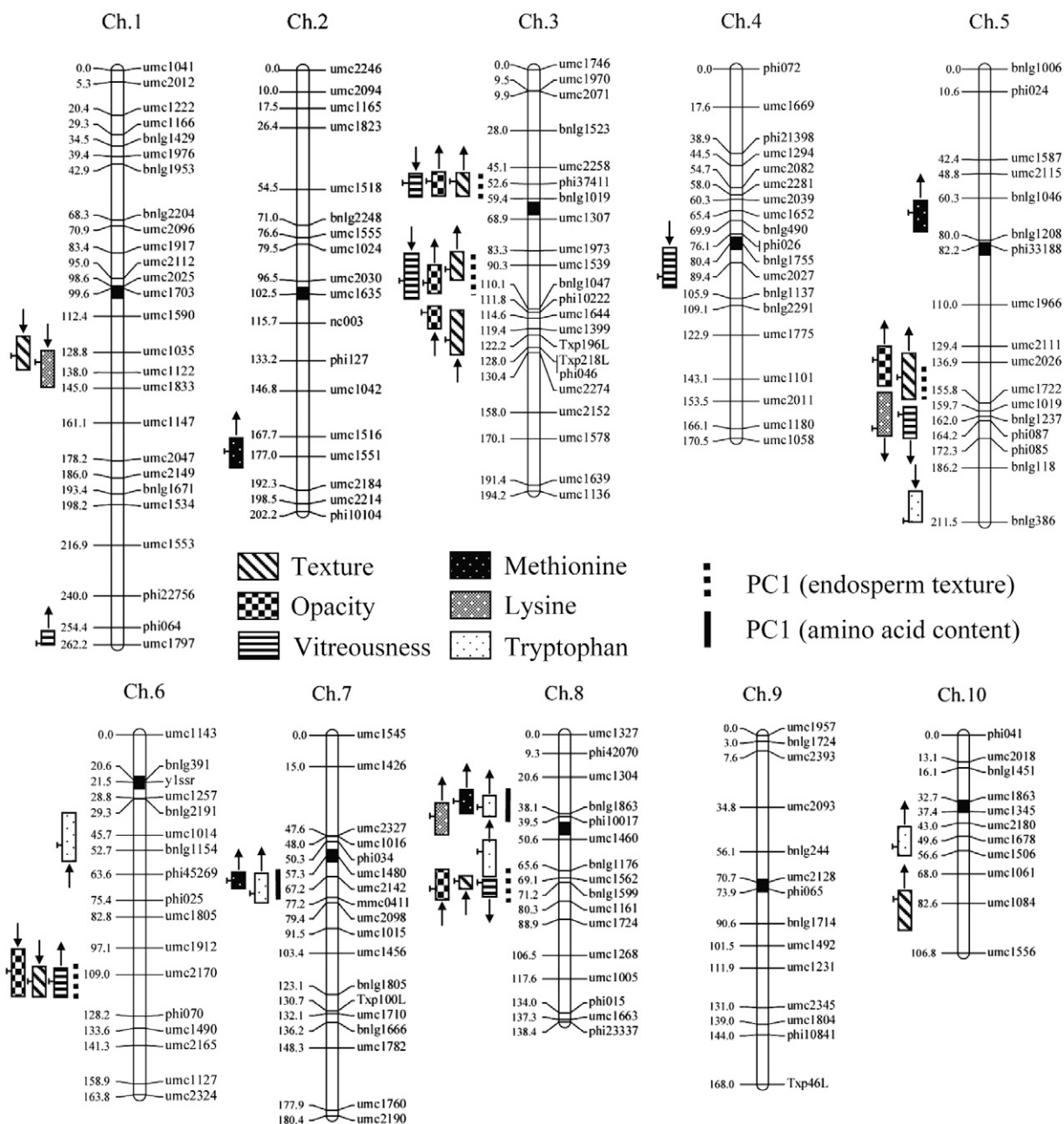


Figure 1. Quantitative trait loci (QTLs) detected with composite interval mapping analysis for traits related with maize endosperm texture modification (texture, opacity, vitreousness, and principal component 1 [PC1]), amino acid contents (lysine, tryptophan, methionine, and PC1) in a population of recombinant inbred lines derived from the cross between maize lines CML161 and B73o2 evaluated in Texas during 2004 to 2006. Arrows indicate the direction of B73o2 allele phenotypic effect (up, increasing; down, decreasing). The length of the QTL boxes depicts the 1-LOD (logarithm of the odds) support interval.

content of methionine came from B73o2. The QTL in bin 7.02 (umc2142) explained 20.7% of the observed variation.

The first two PCs accounted for 90% (PC1 = 73.9% and PC2 = 15.9%) of the variation in the three amino acid content traits. Quantitative trait locus mapping for PC1 identified two major QTLs (Fig. 1 and Table 3). These two QTLs co-localized with clusters of QTLs detected for at least two of the three amino acid-related traits. There was a QTL cluster on chromosome 7L (bin 7.02–7.03), which contains QTLs for methionine and tryptophan. There was a second QTL cluster in chromosome arm 8S (bins

8.02–8.04), which contains QTLs for all three amino acids. In both cases the favorable allele was derived from B73o2.

DISCUSSION

Several of the efforts to improve the nutritional value of maize take advantage of the effect of *o2* mutant alleles in reducing the proportion of the low-quality zeins in the endosperm. The genetic background of the *o2* genotypes must be carefully selected to ensure the presence of modifier loci that overcome the undesirable effects caused by the *o2* allele. The genetic mechanisms behind the modification

Table 2. Quantitative trait loci (QTLs) identified by composite interval mapping (CIM) analysis across environments for the traits related to maize endosperm texture modification: texture (TEXT), opacity (OPAC), vitrousness (VITR), and principal component 1 (PC1) for endosperm texture. QTL peaks or chromosome position with the highest logarithm of the odds (LOD) value, closest marker to the peak, and the bin of the marker according to the consensus maize map are shown.

Trait	Bin	Marker	QTL peak	QTL interval [‡]	LOD	QTL effect [§]	R ² [¶]
							%
TEXT	1.06	umc1035	132.81	123–137	5.00*	−0.11	5.1
	3.02	phi374119	52.6	49–57	5.18*	0.17	7.8
	3.05	umc1539	93.28	85–103	3.91*	0.09	5.2
	3.07	Txp196L	124.25	113–129	2.60	0.08	4.8
	5.05	umc2026	143.92	133–153	3.90*	0.15	8.2
	6.06	umc2170	115.03	106–121	8.84*	−0.30	23.1
	8.05	umc1562	70.06	69–71	10.62*	0.25	19.5
	10.06	umc1061	81	73–93	2.50 [†]	0.09	3.8
	3.02	phi374120	52.6	48–59	2.88 [†]	0.18	5.7
	OPAC	3.05	umc1539	98.28	90–105	5.50*	0.21
3.06		phi102228	113.78	111–119	2.90 [†]	0.12	6.2
5.05		umc2111	136.42	130–150	3.66*	0.20	7.8
6.06		umc1912	107.12	102–119	7.93*	−0.26	13.4
8.05		bnlg1599	74.2	66–78	6.04*	0.27	17.3
1.11		phi064	261.38	256–261	2.81 [†]	1.78	0.6
VITR	3.02	phi374121	53.6	50–57	4.97*	−2.84	9.3
	3.05	umc1539	98.28	86–106	2.87 [†]	−2.54	11.6
	4.06	umc2027	94.38	82–102	3.05 [†]	−1.93	3.7
	5.05	bnlg1237	161.97	157–172	4.49*	−2.33	7.8
	6.06	umc2170	113.03	108–118	6.35*	4.55	27.5
	8.05	bnlg1599	71.2	70–76	5.82*	−2.73	11.4
PC1	3.02	phi374118	52.6	50–57	4.85*	0.49	7.8
	3.05	umc1539	96.28	85–104	4.46*	0.41	9.9
	5.05	umc2026	144.92	138–151	5.87*	0.63	15.3
	6.06	umc2170	114.03	106–120	7.80*	−0.80	23.1
	8.05	umc1562	70.06	68–75	10.34*	0.73	21.4

*QTLs with LOD value above the threshold obtained after 1000 permutations for a significance level = 0.05.

[†]QTLs with LOD value above the threshold obtained after 1000 permutations for a significance level = 0.1.

[‡]The QTL support interval corresponded to an interval on either side of the QTL peak that corresponded to a decrease of 1 LOD from the maximum LOD score.

[§]The QTL effect of the allele derived from B73o2.

[¶]Percentage of the phenotypic variance explained by the QTL estimated by creating a multiple interval mapping model with significant QTLs detected by CIM.

of the endosperm are complex and involve additive, dominant, and recessive gene action, as well as paternal (i.e., xenia) and maternal cytoplasm effects (Lopes and Larkins, 1995; Vasal et al., 1980; Wessel-Beaver and Lambert, 1982).

One of the key steps for understanding the genetic mechanisms of endosperm modification is to locate and identify the genes involved. Previous studies have aimed to map the location of the modifier genes of *o2*. Lopes et al. (1995) used BSA of opaque and modified F₂ individuals selected from two populations visually scored for opacity. They reported one marker (npi277, bin 7.01–7.02), that was linked to kernel modification in one population

and another (umc35, bin 7.05) linked to modification in the second population. Holding et al. (2008) also using BSA of visually selected individuals from two F₂ populations derived from crosses of QPM by *o2* reported markers linked to modifier loci in bins 7.02, 9.03, 9.04, and 9.05; and in bins 6.03, 6.04, 6.05, and 7.02 in the first and second population, respectively. They also reported two major QTLs in bins 7.02 and 9.04 to 9.05 through single-marker analysis and single-kernel phenotyping of kernels from an F₂ ear of one of these populations.

In this work, we have identified five major QTL clusters on chromosomes 3S, 3L, 5L, 6L, and 8L controlling traits related to endosperm texture modification in a multi-environment experimental design that included data from two locations and 3 yr. The percentage of the phenotypic variation explained by the QTL clusters together ranged between 57 and 68% depending on the trait. Each trait corresponded to a different approach to measuring the variation present among the RILs. Common QTLs were expected for these traits due to the high phenotypic ($r = 0.66–0.78$) and genetic correlations ($r = 0.81–1$) between them (Gutierrez-Rojas et al., 2008). The identification of QTL clusters was confirmed by QTL mapping of the largest PC, which explained 87.8% of the variation.

With the exception of the reported QTL on 6L, there were no QTLs in common between this and all previous studies. This can be explained by differences in the genetic background of the populations, by sampling effects associated with QTL mapping in populations of limited size (Beavis, 1998), and by differences in the methods for measuring the phenotype and analyzing the data. Nevertheless, the use of a population of RILs, with homozygous triploid endosperm genome, offers several advantages over other experimental populations (e.g., F₂) used to map modifier genes in the past (Knapp and Bridges, 1990). In a population of RILs, no dominance effects are expected, and the additional rounds of recombination in the development of the lines generates a higher map resolution of the detected QTLs (Lee et al., 2002). In addition, by using RILs we were able to evaluate the population in six environments with replicates, which allowed us to estimate least square means and BLUPs to exert control over the nongenetic variation. The use of different methodologies to evaluate endosperm modification and the application of principal component analysis to analyze correlated traits allowed us to avoid confounding effects caused by the observer (in the case of visual scores) or the measurement technique, and to gain precision in quantifying the variation in endosperm modification. Finally, we also confirmed that both the *o2* and the QPM inbred lines were carrying a copy of the same *o2* mutant allele (Cruz-Vela et al., 2007), so the potential effects of more than one *o2* allele segregating in the population were eliminated.

Our results agree with previous work that has suggested that the modification of *o2* endosperm is quantitative

with polygenic control (Wessel-Beaver and Lambert, 1982). In four of the QTL clusters the source of the modifier allele, which increased the proportion of hard endosperm, was the QPM parent as expected. In the QTL on chromosome 6L, the favorable allele originated from the non-QPM parent. It is possible that B73 contains alleles that increase the proportion of hard endosperm, but once the *o2* recessive allele is introgressed, the effect of these genes is masked. Some endosperm-related genes mapped to this chromosome region are *starch synthase IIa (ssIIa)* or *Sugary2 (Su2)* (bin ~6.04), *Opaque-14 (O14)* (bin ~6.04), and *Pyruvate orthophosphate dikinase1 (cyPpdk1)* (bin 6.05) (Lawrence et al., 2007). Among these genes, special attention has been recently given to *CyPpdk1*. *CyPpdk1* encodes pyruvate orthophosphate dikinase, a protein involved in the control of starch-protein balance in the maize kernel and with an epistatic relationship with *O2* (Prioul et al., 2008).

In *o2* genotypes the content of the limiting amino acids lysine and tryptophan are increased, together with histidine, aspartate, asparagine, and glycine. On the contrary, glutamate, glutamine, alanine, and leucine contents tend to decrease, whereas methionine levels seem to vary independently (Glover and Mertz, 1987). Contradictory reports have described positive (Bantte and Prasanna, 2004), negative (Robutti et al., 1974), or insignificant correlations (Pixley and Bjarnason, 2002) between the level of endosperm modification and amino acid content. The effects of the endosperm texture modification on protein quality seem to be greatly affected by the genetic background (Bantte and Prasanna, 2004). In general, modified *o2* endosperms have a lower proportion of lysine than unmodified *o2* endosperms, but the increase in protein content compensates the loss (Robutti et al., 1974). Modified *o2* genotypes have been found having more tryptophan and less methionine than normal inbreds (Scott et al., 2004; Bantte and Prasanna, 2004). Lysine and methionine are synthesized in the same metabolic pathway, having aspartate as their common precursor (Azevedo et al., 2006). Tryptophan is synthesized from chorismate in a pathway that also provides precursors for important secondary metabolites like hormones and phytoalexins (Radwanski and Last, 1995). Though the metabolic pathways for the biosynthesis of these amino acids are well understood (Azevedo et al., 2006), literature regarding the elucidation of genetic control of variation of amino acid contents in maize is scarce. Wang and Larkins (2001) detected QTLs on chromosomes 2L, 2S, 3S, and 7L controlling free amino acid (FAA) contents in an F_2 population derived from two *o2* lines differing in FAA contents. Later, Wang et al. (2007) used QTL mapping models that take into account the triploid nature of the endosperm and found 11 QTLs in all 10 chromosomes for FAA contents in the same population. Further research has shown that *Ask2*, one of two aspartate kinases cloned in maize, is

Table 3. Quantitative trait loci (QTLs) identified by composite interval mapping (CIM) analysis across environments for the content of amino acids lysine (Lys), tryptophan (Trp), methionine (Met), and principal component 1 (PC1) for amino acid content. QTL peaks or chromosome position with the highest logarithm of the odds (LOD) value, closest marker to the peak, and the bin of the marker according to the consensus maize map are shown.

Trait	Bin	Marker	QTL peak	QTL interval [‡]	LOD	QTL effect [§]	R ² [¶]
							%
Lys	1.06	umc1035	133.81	129–144	3.33*	–1.53	12.0
	5.06	phi087	168.22	150–171	4.27*	–1.07	6.1
	8.03	bnlg1863	38.14	32–47	6.51*	1.60	14.8
Trp	5.07	bnlg118	211.17	197–211	2.98 [†]	–0.28	5.0
	6.04	umc1014	50.7	36–57	2.20	0.27	4.7
	7.02	umc2142	74.2	65–78	3.86*	0.31	7.9
	8.02	umc1304	33.59	29–39	5.45*	0.31	9.5
	8.04	umc1460	59.57	52–71	4.16*	0.23	5.6
	10.04	umc1678	52.57	45–56	5.85*	0.51	16.4
Met	2.08	umc1516	174.69	168–182	3.82*	1.99	9.4
	5.03	bnlg1046	67.32	60–76	3.76*	2.04	8.9
	7.02	umc2142	67.2	63–70	8.91*	2.46	20.7
PC1	8.02	umc1304	32.59	27–38	5.07*	2.44	18.3
	7.02	umc1480	66.27	62–73	5.48*	0.47	12.0
	8.02	umc1304	34.59	28–39	6.20*	0.65	21.2

*QTLs with LOD value above the threshold obtained after 1000 permutations for a significance level = 0.05.

[†]QTLs with LOD value above the threshold obtained after 1000 permutations for a significance level = 0.1.

[‡]The QTL support interval corresponded to an interval on either side of the QTL peak that corresponded to a decrease of 1 LOD from the maximum LOD score.

[§]The QTL effect of the allele derived from B73o2.

[¶]Percentage of the phenotypic variance explained by the QTL estimated by creating a multiple interval mapping model with significant QTLs detected by CIM.

tightly linked to the QTL on chromosome 2L that affects FAA content (Wang et al., 2007). In this study, we found significant differences between the inbred parents B73o2 and CML161 for lysine, tryptophan, and methionine content, with the *o2*-modified QPM inbred being inferior for all three amino acids. Heritability estimates for the content of these three amino acids were high ($H_{\text{mean basis}} = 0.71–0.80$) (Gutierrez-Rojas et al., 2008); consequently, QTLs for the contents of lysine, tryptophan, and methionine were detected in the population of RILs. Quantitative trait locus mapping of the largest PC explaining 73.9% of the phenotypic variation for lysine, tryptophan, and methionine identified two QTL clusters on chromosomes 7L and 8S. This last QTL cluster on 8S explained variation of the levels of lysine, tryptophan, and methionine, and because these three amino acids are synthesized by two different pathways, it is possible that these QTLs control total protein content. A QTL for grain protein content was identified at a similar position in a previous study (Melchinger et al., 1998). Other QTLs with major effects were mapped on several other chromosomes, with favorable alleles increasing the amount of amino acids in the endosperm derived from both parents.

Possible candidates for genes controlling amino acid levels are those involved in the biosynthetic pathways of the amino acids studied. For example, four QTLs controlling methionine content were identified. Those in bins 2.08 and 5.03 map close to the positions of bacterial artificial chromosome (BAC) contigs (2.05 and 5.02) that were detected by hybridization using a probe corresponding to methionine synthase (Lawrence et al., 2007). Similarly, an overgo probe containing a putative anthranilate synthase gene hybridized to BACs in bin 10.04, co-localizing with the QTL explaining the most phenotypic variation for lysine, tryptophan, and methionine in our study (Lawrence et al., 2007). The QTL cluster identified in bin 7.02 for tryptophan, methionine, and PC1 is mapped close to the position of the *o2* gene; however, based on sequence and expression data *o2* should not be segregating in this population, so it is unlikely that contributes to these QTLs. Potential candidates are an aspartate kinase and a 27-kDa γ -zein gene found in this region. Aspartate kinase is a key enzyme in the methionine biosynthetic pathway and the 27-kDa γ -zein is the single most abundant zein polypeptide.

It was of interest to us to examine if QTLs for endosperm texture modification and amino acid contents co-localized. Overlap of QTLs for two traits might reflect a pleiotropic effect of a single gene, or tight linkage of genes controlling the traits independently. Several reports mention that there is not a strict relationship between protein quality and endosperm texture (Moro et al., 1995). Nonetheless, a general negative correlation between increased endosperm modification (more vitreous and less opaque) and protein quality would create the requirement for simultaneous selection for texture and amino acid contents in the development of the QPMs (Bjarnason and Vasal, 1992; Gutierrez-Rojas et al., 2008). We observed that QTLs explaining variation for endosperm texture and amino acid (lysine, tryptophan, and methionine) content did not overlap along the genetic map (Fig. 1). Nevertheless, it is noteworthy that a cluster that contains QTLs for the three amino acids and a cluster that contains QTLs for the three endosperm texture traits are in close proximity on chromosome 8 (Fig. 1). Some endosperm-related genes located in this genomic regions are the *b32 ribosome inactivating protein (b32/RiP)* (bin 8.03), *glycer-aldehyde-3-phosphate dehydrogenase1 (gpa1)* (bin 8.03), *Proline Responding 1 (Pro1)* or *Opaque-6 (O6)* (bin 8.04), *Floury-3 (FL3)* (bin 8.04), *pyruvate orthophosphate dikinase-2 (ppdk2)* (bin 8.04), and *Opaque-16 (O16)* (bin 8.05). The two QTL clusters that were well defined by MIM analysis appear to be on different arms of chromosome 8 and are linked in repulsion. The recombination fraction between the closest markers to the QTL peaks, *bnlg1863* and *umc1562*, is $r = 0.276$. This observation might have breeding implications because selection for the favorable allele controlling endosperm texture could drag the unfavorable allele for amino acid content and reduce the amount of lysine, tryptophan,

and methionine by at least 10%. Additional studies would be needed to establish the prevalence of this linkage drag effect in other populations.

The information generated here can readily be utilized to develop a modified B73*o2* line carrying the most important favorable QTL alleles derived from CML161 (i.e., texture QTLs 3S, 3L, 5L, and 8L) and retain the favorable QTL alleles derived from B73*o2* (texture QTL 6L and amino acid QTLs 7L and 8S). By using SSR markers flanking the QTL regions as markers for selection, maize breeders would ensure the selection of the favorable QTLs and decrease the risk of linkage drag that can diminish its nutritional value.

To our knowledge, this is the first time that both endosperm texture and amino acid contents have been exhaustively evaluated in a multienvironment experiment with a population derived from crossing *o2* and QPM materials. Despite the fact that QTLs identified here do not co-locate with those identified in previous studies and therefore did not provide clear consensus regions for the location of modifiers for the gene *O2*, we are confident that the complexity of the endosperm modification can be unveiled with additional studies. A following step of this work could be the development of near isogenic lines (NILs) using an *o2* genotype (i.e., B73*o2*) as recurrent parent with QPM CML161 donor alleles to establish the effects of the individual QTLs for endosperm texture on chromosomes 3S, 3L, 5L, and 8L. A similar approach can be used to establish the effects of the QTL cluster on 6L using B73*o2* as the donor parent. The development of NILs will contribute to improved resolution of the QTL regions and facilitate the identification of candidate genes by fine-mapping. Moreover, results of a complementary expression QTL (eQTL) mapping study to analyze genetic control of mRNA abundance of endosperm transcripts (e.g., zein genes, enzymes in the starch biosynthesis, elongation factor 1A, etc.) suggest a role for differential regulation of some endosperm genes in the modification of *o2* endosperm (A. Gutierrez-Rojas, J. Betrán, C. Cruz-Vela, and M. Menz, unpublished results, 2008). In addition, by developing new mapping populations or by ongoing multipopulation studies, such as the Nested Association Mapping experiment (Buckler, 2006), it would be possible to test the prevalence of the effects of these genomic regions on the amino acid composition and endosperm structure of different genetic backgrounds.

CONCLUSIONS

After the enthusiasm in the late 1960s and early 1970s for the use of the *o2* alleles in breeding programs, there was a general disappointment when the undesirable traits associated with the mutation were identified. However, the discovery of the effect of the *o2* in protein quality spurred research on the biochemistry, physiology, microstructure, and genetics of endosperm of maize and other crops like

sorghum [*Sorghum bicolor* (L.) Moench], rice (*Oryza sativa* L.), and wheat (*Triticum aestivum* L.). The development of the QPMs has demonstrated that a positive response to selection can be obtained for both endosperm texture and amino acid contents with simultaneous selection schemes. We have detected QTL regions for both endosperm modifications and essential amino acid contents. Accumulated evidence suggests that modification of the endosperm in *o2* maize is complex with several genetic factors playing roles in both endosperm texture and amino acid content, with the genetic background being one of the most important variables contributing to the variation observed so far. The broader applicability of this accumulating knowledge to plant breeding programs depends on enhanced understanding of the phenotypes resulting from the interaction between the *Opaque2* gene and different genetic backgrounds.

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