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ORIGINAL PAPER

Characterization of *opaque2* modifier QTLs and candidate genes in recombinant inbred lines derived from the K0326Y quality protein maize inbred

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Abstract Quality protein maize (QPM) is a high lysine-containing corn that is based on genetic modification of the *opaque2* (*o2*) mutant. In QPM, modifier genes convert the starchy endosperm of *o2* to the vitreous phenotype of wild type maize. There are multiple, unlinked *o2* modifier loci (*Opm*) in QPM and their nature and mode of action are unknown. We previously identified seven *Opm* QTLs and characterized 16 genes that are differentially up-regulated at a significant level in K0326Y QPM, compared to the starchy endosperm mutant W64A*o2*. In order to further characterize these *Opm* QTLs and the genes up-regulated in K0326Y QPM, we created a population of 314 recombinant inbred lines (RILs) from a cross between K0326Y QPM and W64A*o2*. The RILs were characterized for three traits associated with endosperm texture: vitreousness, density and hardness. Genetic linkage analysis of the RIL population confirmed three of the previously identified QTLs associated with *o2* endosperm modification in K0326Y QPM. Many of the genes up-regulated in K0326Y QPM showed substantially higher levels of expression in vitreous compared with opaque RILs. These included genes associated with the upstream regulation of the ethylene response pathway, and a gene encoding a regulatory

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subunit of pyrophosphate-dependent fructose-6-phosphate 1-phosphotransferase, an adaptive enzyme of the glycolytic pathway.

Introduction

The maize *opaque2* (*o2*) mutation increases the lysine content in the endosperm by decreasing the synthesis of zein storage proteins and increasing the synthesis of other types of proteins. While this improves the nutritional quality of the grain, the low seed density and soft texture associated with *o2* lead to brittleness and insect susceptibility, thereby decreasing the value of the grain. These negative features of the mutant can be ameliorated by *o2* modifier (*Opm*) genes, which create a normal kernel phenotype while maintaining a high lysine content. There are multiple *Opm* loci with complex phenotypic effects (Belousov 1987; Vasal et al. 1980; Geevers and Lake 1992). By introgressing these genes into *o2* backgrounds, plant breeders were able to create an improved *o2* version, which was called quality protein maize (QPM) (Prasanna et al. 2001; Gibbon and Larkins 2005).

The complexity of introgressing multiple, unlinked *Opm* loci has slowed the creation and widespread use of QPM. This process could be accelerated if the chromosomal locations of these genes and the mechanism(s) by which they create a vitreous kernel were known. We recently identified the location of QTLs associated with *o2* endosperm modification in two different QPM backgrounds: Pool 33, developed by CIMMYT in Mexico (Vasal et al. 1980; Bjarnason and Vasal 1992) and K0326Y, developed in South Africa (Geevers and Lake 1992). Both QPMs were crossed to W64A*o2*, a starchy endosperm Midwestern inbred, and F2/F3 progeny segregating for a vitreous or starchy kernel phenotype were screened for *Opm* QTLs by bulked segregant analysis (BSA) (Michelmore et al. 1991) using SSR DNA markers (Holding et al. 2008). We also phenotyped and genotyped single F2 kernels of the K0326Y QPM × W64A*o2* cross. Three *Opm* QTLs were identified for each of the segregating populations: Pool 33 QPM revealed QTLs in bins 6.03, 6.04, and 7.02, while the K0326Y QPM had QTLs in bins 7.02, 9.03, and 9.05. Results of the single seed analysis of F2 progeny from the K0326Y QPM × W64A*o2* cross confirmed and expanded these data: seven *Opm* QTLs were identified that accounted for approximately 75% of the phenotypic variation. Loci in bins 7.02 and 9.04/9.05, which corresponded to the QTLs identified by BSA, accounted for 40% of the phenotypic variation. QTLs in bins 1.05/1.06, 1.07/1.08, 5.01/5.02, and 10.02 contributed 28% of the phenotypic variation.

In the above study, we also identified genes that are differentially expressed at 18 DAP in the endosperm of K0326Y QPM and W64A*o2*. This analysis identified 16 genes that were differentially regulated in K0326Y QPM and the vitreous progeny generated from the cross with W64A*o2*. The map position of these genes revealed that several of them co-localize in the bins linked with *Opm* QTLs on chromosomes 1, 6, 7, and 9. Thus, several of these genes were considered candidates for *Opm* QTLs.

In order to analyze the effects of individual *Opm* QTLs and create genetic stocks suitable for map-based cloning of these genes, we created a population of recombinant inbred lines (RILs) from the K0326Y QPM by W64A*o2* cross. Each RIL was derived by single seed descent from kernels of a single well-filled F2 ear. After seven generations of self-pollination, 314 RILs were recovered that showed a range of phenotypes from vitreous to opaque. The RILs were phenotyped for vitreousness, density, and hardness and genotyped with the SSR markers used to characterize the F2 population. Linkage analysis of these traits revealed QTLs that match those previously identified. A number of the genes differentially expressed in K0326Y QPM were found to be highly expressed in the vitreous RILs and were expressed below W64A+ levels in the opaque RILs. Several of these genes are associated with the upstream regulation of the ethylene response pathway, and one encodes the regulatory subunit of pyrophosphate-dependent fructose-6-phosphate 1-phosphotransferase, a non-ATP-requiring enzyme that catalyzes a major rate-limiting step in glycolysis.

Materials and methods

Genetic materials

The K0326Y QPM inbred line (Geevers and Lake 1992) was provided by Dr. Hans Geevers, and W64A*o2* (a starchy endosperm *o2* mutant) was obtained from Purdue University, West Lafayette, Indiana. The nearly isogenic lines CM105+, CM105*o2*, and modified CM105*o2* (CM105*mo2*) were previously described (Gibbon et al. 2003). Recombinant inbred lines (RILs) were derived by a modified single seed descent procedure with 400 kernels from a well-filled F2 ear of a cross between K0326Y QPM and W64A*o2*. Because it was important to have a wide range of intermediate kernel phenotypes between those of the parents, visual selection with a light box was used during the early generations of inbreeding to assure a broad range of phenotypes in the next breeding cycle. Some genotypes were lost over time due to either a lack of fertility or contamination. The S1–S5 RIL generations were grown in Arizona between 2001 and 2004 and the S6–S7 generations were grown at the Pioneer Hi-Bred summer nursery in Johnson, IA, between 2004 and 2005. The zein protein composition of kernels from each S6

RIL was analyzed by SDS–PAGE to verify a homozygous *o2* genotype, based on reduced synthesis of the 22-kDa α-zeins. Developing endosperm used for RNA transcript analysis was obtained 18 days after pollination (DAP) from S7 plants grown at the University of Arizona West Campus Agricultural Center in 2008.

Kernel phenotyping

The density of maize kernels was measured with a Micromeritics AccuPyc II 1330 Gas Pycnometer (Micromeritics Instrument Corporation, Norcross, GA) using 10 cc cups (about 30–50 kernels) according to manufacturer's procedures (purge-gas: helium; calibration: 10 purges, 10 cycles; analysis: 3 purges, 1 cycle). Kernels were obtained from the middle third of an ear and dried 24 h at 70°C in a vacuum oven. For each ear, pycnometer analysis was performed with three randomly selected and weighed (~10 cc) kernel samples and kernel density for the ear was calculated (averaged) from three such measurements. For each RIL, three independent ears were analyzed and the three-ear average was recorded as the RIL true density.

Kernel hardness was based on the Stenvert grinding resistance test, as described by Pomeranz et al. (1985). Kernel samples were obtained from the middle third of an ear and dried in a vacuum oven at 70°C for 24 h. 17 ml of a 20 g sample of maize kernels was ground using a Type V Micro Hammer/Cutter Mill (Glen Mills, Clifton, NJ) fitted with a 2 mm aperture particle screen, a plastic test tube collection port and a speed control tachometer. The mill speed was set to read 360 rpm on the tachometer. Before analytical runs, the mill was allowed to warm up by grinding five-grain samples. The time taken to grind 17 ml of kernels was recorded in seconds.

To measure vitreousness, individual kernels were mounted over small holes cut in 3 × 5 inch index cards and placed on a light box. Light transmitted through the kernel was measured with a digital camera and recorded in arbitrary units using Adobe Photoshop (Adobe, San Jose, CA) (Holding et al. 2008).

Zein protein extraction and analysis

Zein proteins were extracted from mature endosperms and analyzed as described by Wallace et al. (1990). Proteins from 1 mg of endosperm flour were resolved by 12% SDS–PAGE.

DNA preparation and SSR marker analysis

DNA was extracted from leaves of one 15–20 cm tall seedling representing each RIL using the CTAB method (Shen et al. 1994). After precipitation with 2.5 volumes of ethanol and 0.1 volume of 3 M sodium acetate, pH 5.3, and washing with 70% ethanol, the DNA was dissolved in sterile water and the concentration determined with a NanoDrop Spectrophotometer ND-100 (NanoDrop Technologies, Wilmington, DE). The DNA was diluted to approximately 10 ng/μl for PCR reactions. Most DNA primers (Invitrogen, Carlsbad, CA) used for SSR amplification were described by Holding et al. (2008). The SSR sequence linked to the 27-kDa γ-zein gene is about 1,000 bp upstream of the transcription start site and can be amplified with these primers: (5′) CGGTTTCTCCT AAATACTCCCCC and (3′) CTATACCTACGTC CCCACCCTT. The PCR reactions were performed using Taq DNA polymerase and buffer from New England BioLabs (Ipswich, MA), with the addition of 1 M betaine and 2% DMSO, in a Peltier Thermal Cycler PTC-200 (MJ Research, Waltham MA) or an Eppendorf MasterCycler (Eppendorf, Westbury, NY) as described in Holding et al. (2008).

Linkage analysis and QTL detection

Interval mapping was used to scan for the presence of significant QTLs throughout the linkage map common to the RILs. Two predominant homozygotes for each allele of K0326Y QPM and W64A*o2* were considered. The conditional probabilities of a particular QTL genotype given marker genotypes in a RIL population are described in Wu et al. (2007). Permutation tests based on 1,000 replicates were used to determine the critical value that claims the existence of a significant QTL.

Developmental materials

The RILs were sorted using an Excel spreadsheet based on their degree of vitreousness and a small subset selected for molecular analysis. The RILs chosen represent the range of phenotypic variation and differences in chromosomal constitution based on flanking SSRs for the QTLs on chromosomes 1, 7, and 9. Plants were grown at the University of Arizona, West Campus Agriculture Center. After selfing, developing ears were harvested 18 DAP. The kernels were frozen in liquid nitrogen at the field site and stored at −80°C. RNA was extracted as previously described (Holding et al. 2008) and transcript levels determined by qRT-PCR.

Expression of *Opm* candidate genes in the K0326Y QPM × W64A*o2* RILs

Alleles of candidate genes from W64A*o2* and K0326Y QPM were obtained by PCR amplification of full-length or partial cDNA sequences using ultra high fidelity Phusion

polymerase (Finnzymes, Espoo, Finland) from triplicate endosperm RNA samples, cloning into pCR2 TOPO (Invitrogen, Carlsbad, CA), and bi-directional sequencing using M13F and M13R primers. Consensus sequences for W64A*o2* and K0326Y QPM alleles were obtained using CodonCode aligner software. Real-time PCR primers (Table S2) were designed for a 60°C annealing temperature to amplify a 100–200 bp region using the Primer 3 software available at http://frodo.wi.mit.edu/. Gene expression was compared between 12 genotypes: W64A+, W64A*o2*, K0326Y QPM and nine RILs chosen among the phenotypic classes identified as vitreous, semi-vitreous and opaque. Total RNA was extracted from endosperms dissected from six pooled kernels from each of three 18 DAP ears (biological replicates) for each genotype. The cDNA was synthesized from 1 μg samples of DNase 1-treated total RNA using IScript plus (Bio-Rad, Hercules, CA), according to the manufacturer's instructions. The cDNAs were diluted tenfold in water and amplified using iQ SYBR green super mix (Bio Rad, Hercules, CA) according to the manufacturer's instructions. A My iQ Real-time PCR thermocycler (Bio Rad, Hercules, CA) was used with the following program: 95°C for 5 min, followed by 45 cycles of 95°C (10 s) and 60°C (10 s) with 20°C per second ramp rates. Melting curves were obtained by heating from 65°C to 95°C with a 0.1°C per second ramp rate. Expression levels of candidate genes were calculated relative to W64A+ using the RRb1 gene (Sabelli et al. 2005) as a control, since it was repeatedly shown in preliminary experiments not to be differentially expressed between samples. For each gene tested, the average cycle threshold (Ct) value was calculated for the three biological replicate ears of each genotype. The relative expression was calculated using the following equation, where X = gene of interest, C = control gene, RRb1, W = average Ct of three W64A+ ears, G = average Ct of three ears of other genotypes: $2^{[(WX-WC)-(GX-GC)]}$. The final fold-change values shown are the mean of three independent experiments (±standard deviation).

Results

Using a cross between K0326Y QPM × W64A*o2*, we identified seven QTLs that influence the formation of a vitreous kernel phenotype (Holding et al. 2008). In this analysis, we also identified 16 transcripts that appeared to be up-regulated in the vitreous but not the opaque progeny; these genes map coincidentally with QTLs in bins 1.05, 7.02, 7.03, 7.04, 7.05, 9.03, 9.04, and 9.05. To create genetic materials that would allow us to dissect these QTLs and investigate their relationship to the expression of candidate genes, we developed a set of RILs from 400 kernels of a single well-filled F2 ear by single seed descent. To ensure a diverse range of kernel phenotypes was carried forward after each generation, a light box was used to evaluate the phenotypic variability of RIL families during sequential inbreeding cycles. Ultimately, 314 RILs (S7) were created that manifested a broad range of kernel phenotypes.

It is difficult to distinguish the vitreous phenotype of wild type kernels from those of modified *o2* mutants; therefore, it was important to rule out the possibility of wild type pollen contamination of the RILs. Endosperm proteins from each RIL were analyzed by SDS–PAGE to examine the level of 22-kDa α-zeins, as the synthesis of these proteins is controlled by the O2 transcription factor (Kodrzycki et al. 1989). The results of this analysis confirmed that the 22-kDa α-zeins were markedly reduced in all of the RILs (Fig. 1), regardless of their vitreous or opaque phenotype. Therefore, all of the RILs appear to be homozygous recessive at the O2 locus.

We measured three kernel phenotypes to assess the degree of *o2* endosperm modification: vitreousness, hardness, and density. As shown in Table S1, the RILs had a broad range of vitreousness (based on light transmission measured with a digital camera), with approximately 50-fold variation from the most vitreous to the most opaque. There was also a large degree of variation in density, 1.10–1.37 g/ml, and a tenfold variation in hardness as measured by the Stenvert grinding resistance test. These three traits are thought to be related (Mestres and Matencio 1996), and we performed regression and correlation analyses for them in the RIL population. A good correlation was observed between vitreousness and density ($R = 0.71$) and between vitreousness and hardness ($R = 0.62$) (Table 1). Hardness and density were not as highly correlated ($R = 0.51$) (Table 1) as the other two traits, but all three correlations were found to be statistically significant by ANOVA ($p < 0.0001$).

The RILs were genotyped with the same set of 140 informative SSR markers we previously used to analyze the F2 progeny of the cross between K0326Y QPM and W64A*o2* (Holding et al. 2008). Based on the linkage maps

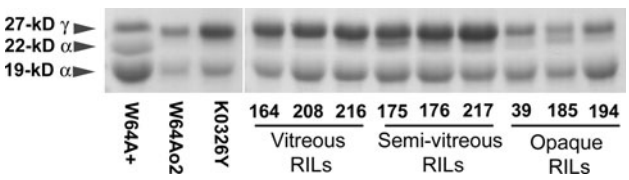

Fig. 1 Profiles of the 27-kDa γ-zein and 22- and 19-kDa α-zein storage proteins in mature endosperms of W64A+, W64A*o2*, K0326Y QPM and selected K0326Y QPM × W64A*o2* RILs. Each *lane* represents the ethanol-soluble proteins extracted from 1 mg (dry weight) of endosperm flour

Table 1 Relationships between vitreousness, density and grinding time (hardness) traits among the recombinant inbred lines

	Correlation (*R*)	95% CI	ANOVA
Vitreousness and density	0.71	[0.65, 0.76]	$p < 0.0001$
Vitreousness and hardness	0.61	[0.53, 0.67]	$p < 0.0001$
Hardness and density	0.51	[0.42, 0.59]	$p < 0.0001$

of the RILs, we identified QTLs at the 1% genome-wide significance level for vitreousness, hardness, and density. QTLs affecting vitreousness were found on chromosomes 1, 7, and 9 that explained approximately 6, 38, and 12%, respectively, of the phenotypic variation for this trait (Fig. 2a, Table 2). Among these, the one on chromosome 7 had the largest additive effect, followed by those on chromosomes 9 and 1. Two QTLs affecting density were detected on chromosomes 7 and 9 that explained 38 and 12%, respectively, of this phenotypic variation (Fig. 2b). Both of these QTLs had the same flanking markers as those associated with vitreousness (Table 2). Only one QTL affecting hardness was found; it was on chromosome 7 (Fig. 2c) and shared the same flanking markers as the QTL associated with vitreousness and density (Table 2). This QTL accounted for 23% of the phenotypic variation for this trait. For each QTL, the K0326Y QPM parent contributed the favorable allele for increasing the value of each trait. The QTL on chromosome 7 appears to be pleiotropic, as it influences all three traits, while the QTL on chromosome 9 affects vitreousness and density.

Expression of *Opm* candidate genes in the parental inbreds and selected RILs

The *Opm* candidate genes we previously described were identified based on differential hybridization of RNAs in microarray comparisons of pools of vitreous and opaque progeny obtained from the K0326Y QPM by W64A*o2* cross (Holding et al. 2008). In the present study, we designed primers for real-time PCR based on regions of 100% sequence identity between cDNAs corresponding to the genes differentially expressed in K0326Y QPM and W64A*o2* endosperms. The purpose of this analysis was to verify the differential expression of these genes and address three questions: (1) How do the expression levels of these genes in starchy and modified *o2* endosperms compare to wild type—Is the expression reduced in starchy *o2* and restored to wild type levels in K0326Y QPM, or are the expression levels in QPM greater than wild type? (2) How do expression levels of these genes in the parental inbreds (W64A*o2* and K0326Y QPM) compare with those in the true-breeding progeny (F4s) used in the microarray experiment? (3) What is the level of expression in the vitreous and opaque RILs developed from the K0326Y QPM × W64A*o2* cross? For this analysis we used three each of the vitreous, semi-vitreous and opaque RILs

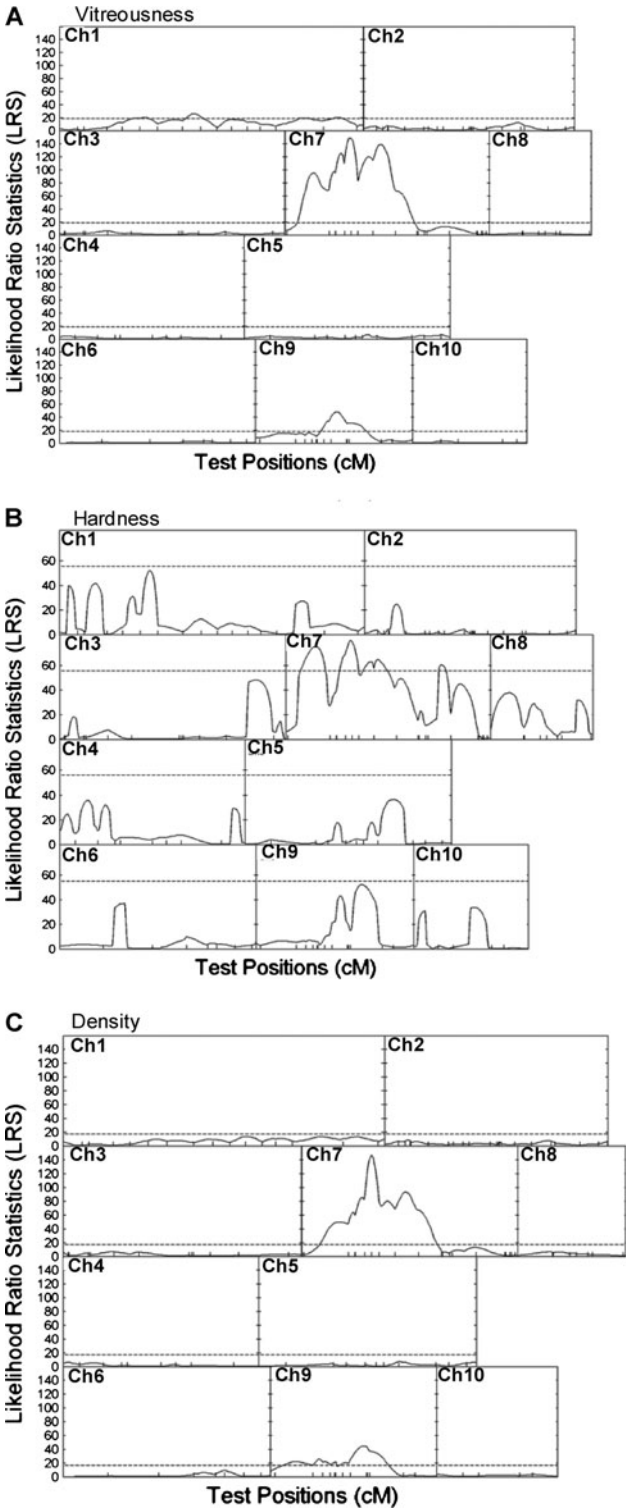

Fig. 2 The plot of log-likelihood ratios for: **a** vitreousness; **b** hardness; and **c** density throughout the entire linkage map of the K0326Y QPM by W64A*o2* cross. The *horizontal line* is the genome-wide critical value at the 1% significance level obtained from 1,000 permutations

Table 2 Chromosomal position of QTLs and their additive (*a*) genetic effects and proportions (R^2) of the phenotypic variance

Chromosome-trait	Marker interval	*a*	% Variance (R^2)
Vitreousness			
1	umc1076–umc1335	15.31	6.3
7	umc1036–γ zein	29.69	38.0
9	bnlg127–umc1771	21.21	12.4
Breakage mean			
7	umc1036–γ zein	79.22	22.7
Density			
7	umc1036–γzein	0.04	37.8
9	bnlg127–umc1771	0.025	13.0

(Fig. 3, Table 3), along with W64A+, W64A*o2*, and K0326Y QPM. Endosperm RNA was extracted from 18 DAP developing kernels, and real-time PCR was used to test for differential expression with 10 of the 16 previously identified differentially expressed genes. These ten genes were selected based on their map position relative to QTLs, their predicted function and the magnitude of expression difference in the microarray hybridization. The W64A*o2* and KO326Y QPM gene sequences were determined for all ten genes to ensure real-time PCR primers were designed to invariant regions. Of the ten genes examined, differential expression was confirmed for six of them. These results, along with those for an additional ethylene response-related gene (*ZmERF1*), are shown in Table S3 and Fig. 4.

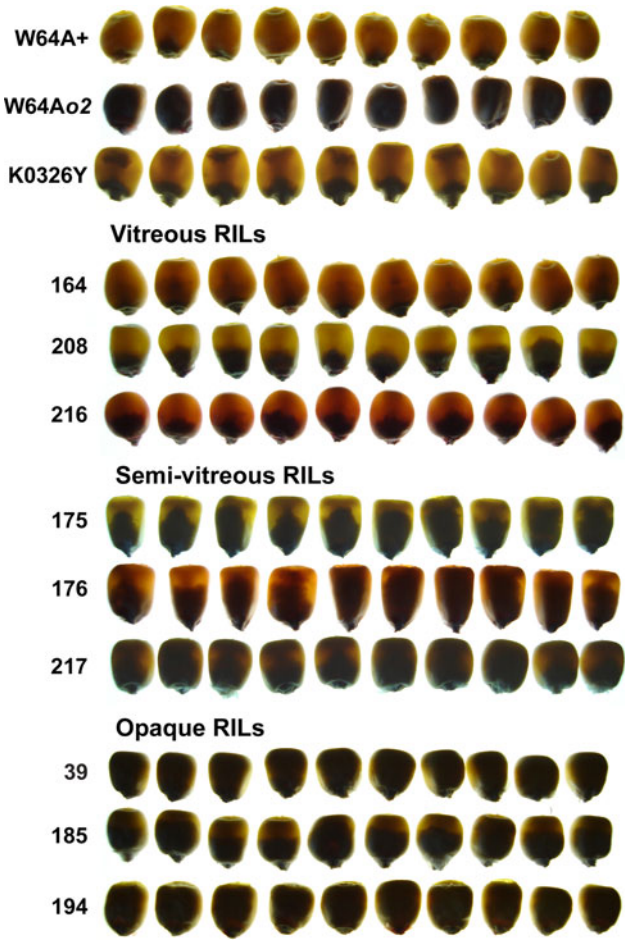

Fig. 3 Kernel phenotypes of W64A+, W64A*o2*, K0326Y QPM and selected K0326Y QPM × W64A*o2* RILs. 10 kernels from representative ears were illuminated on a *light box* and photographed using a digital camera

As shown in Fig. 4a, 27-kDa γ-zein gene expression was 2.1-fold greater in K0326Y QPM than W64A*o2*. In the vitreous and semi-vitreous RILs, 27-kDa γ-zein expression was between ~1.5- and ~3.0-fold increased compared with W64A*o2*, and the opaque RILs had even lower 27-kDa γ-zein gene expression than W64A*o2*. Although the increased expression of 27-kDa γ-zein transcripts was variable among the vitreous and semi-vitreous RILs, there was a consistently elevated level of 27-kDa γ-zein protein among them (Fig. 1). These data support previous studies linking increased expression of the 27-kDa γ-zein protein with modification of the *o2* phenotype, and suggest that this is at least partially regulated at the transcriptional level (Geetha et al. 1991).

The glucose transporter gene that maps close to the QTL for vitreousness on chromosome 7 was determined to be approximately twofold up-regulated in vitreous compared to opaque pools (Holding et al. 2008). Here we found that expression in W64A*o2* is approximately twofold reduced with respect to W64A+, and that expression in K0326Y QPM is similar to the W64A+ level (Fig. 4b). There appears to be a good correlation between the expression of this gene and the degree of modification in the RILs, since the vitreous and semi-vitreous RILs showed W64A+ or greater expression, whereas the expression in the opaque RILs more closely matched that in W64A*o2*.

The type 2C protein phosphatase gene, similar to ABI1 and ABI2 of Arabidopsis (Hirayama and Shinozaki 2007), showed a dramatic (56-fold) up-regulation in the vitreous versus the opaque pools (Holding et al. 2008). Using allele-common primers, we found that expression of this gene in K0326Y QPM is 45-fold increased with respect to W64A+, and there is an approximate twofold down-regulation in W64A*o2* (Fig. 4c). Such a dramatic up-regulation of this gene was not observed in the RILs, although its expression was generally higher than in W64A+ and W64A*o2*. The vitreous RILs showed significantly higher expression than the three semi-vitreous RILs and two of the three opaque RILs. But one opaque RIL, 194, had substantially higher expression (a 6.5-fold increase compared to W64A+). These results do not rule out the involvement of the type 2C phosphatase in *o2* modification, especially if it acts in conjunction with the products of other modifier genes.

Table 3 Phenotypic and genotypic characterization of recombinant inbred lines selected for profiling of genes differentially expressed in K0326Y QPM versus W64A*o2*

RIL #	Phenotype[a]			QTL flanking markers[b]					
	Vitreous	Breakage	Density	Chromosome 1		Chromosome 7		Chromosome 9	
164	87.6	409.6	1.302	B	B	A	A	A	B
208	112.7	382.6	1.317	A	A	B	A	A	A
216	91.4	380.8	1.320	B	B	B	A	B	B
175	90.1	319.3	1.321	A	A	B	A	B	B
176	32.3	314.4	1.229	A	A	B	A	B	B
217	50.8	344.0	1.273	A	A	B	A	B	A
39	7.1	225.1	1.190	A	A	B	B	B	B
185	10.4	198.5	1.154	B	B	B	B	B	B
194	12.5	167.0	1.182	B	B	B	B	B	B

[a] The methods for determining the degree of vitreousness, breakage (hardness) and density are described in "Materials and methods"

[b] A and B designate the SSR alleles from K0326Y (A) and W64A*o2*, (B) that flank QTLs identified on chromosomes 1, 7 and 9

One of the most highly up-regulated genes in K0326Y QPM and the vitreous pools derived from its cross with W64A*o2* encodes the regulatory α-subunit of pyrophosphate-dependent fructose-6-phosphate 1-phosphotransferase, also known as PFP (Holding et al. 2008). This gene referred here as PFPα, maps to bin 9.03 and was up-regulated between 5.2-fold (microarray) and 16-fold (real-time PCR) in the vitreous pools (Holding et al. 2008). Using allele-common PCR primers, we found that PFPα is up-regulated 17-fold with respect to W64A+, and furthermore it is more than tenfold down-regulated in W64A*o2* (Fig. 4d). PFPα was highly expressed in most of the vitreous and semi-vitreous RILs, and the opaque RILs all showed down-regulation of PFPα, similar to W64A*o2*. This suggests that high expression levels of this gene can contribute to the formation of a vitreous kernel, but it is not the only mechanism leading to this phenotype.

An EIN3-like (EIL) gene that maps close to the QTL in bin 9.05 was also found to be up-regulated in the vitreous pools derived from the K0326Y QPM by W64A*o2* cross (Holding et al. 2008). Based on the microarray experiment, EIL was 3.3-fold up-regulated in vitreous pools, but the real-time PCR data suggested a smaller increase (1.4-fold). This discrepancy is likely explained by the fact that after sequencing this gene in the W64A*o2* and K0326Y QPM backgrounds, we found several polymorphisms in the K0326Y QPM primer binding sites. Using primers common to the K0326Y QPM and W64A*o2* alleles, we found that the EIL gene in K0326Y QPM has markedly higher expression compared to W64A+ (41-fold) and W64A*o2* (38-fold). Like PFPα, a similarly high expression level of EIL was observed in the same two (164 and 208) vitreous RILs. The three semi-vitreous RILs also showed elevated EIL expression relative to wild type, although it was much less pronounced (Fig. 4e). The opaque RILs generally had low EIL expression, similar to or slightly greater (1.1- to 2.6-fold) than W64A+. Therefore, expression of EIL appears to be closely related to the degree of endosperm modification. Noteworthy is the fact that EIL expression in K0326Y QPM and the vitreous RILs is elevated over both W64A+ and *o2*, unlike the glucose transporter where expression was restored to a wild type level.

Since EIN3 is a key upstream regulator in the ethylene response pathway (Guo and Ecker 2004), we examined whether genes involved in ethylene signaling respond to the increased expression of EIL in K0326Y QPM and the RILs. We tested the expression of two putative downstream ethylene response factors. The first was identified as a putative ethylene responsive element binding protein (EREBP) in the same BAC contig as the 27-kDa γ-zein gene on chromosome 7.02, and was shown to be 2.6-fold up-regulated in vitreous compared with opaque pools (Holding et al. 2008). An analysis of the RILs suggested that the EREBP gene is approximately fourfold up-regulated with respect to both W64A+ and *o2* (Fig. 4f), in agreement with the previous results (Holding et al. 2008). Interestingly, the expression of this gene was variably increased in all nine RILs, compared with W64A*o2* and wild type, irrespective of phenotype. Expression in two of the vitreous RILs (164 and 208) and two of the semi-vitreous RILs (175 and 176) was even higher than in K0326Y QPM.

The second downstream ethylene response factor we tested is the putative maize ortholog (Genbank accession # Y672654) of the Arabidopsis *Ethylene Response Factor 1* (*ERF1*) gene, a direct target of EIN3 (Solano et al. 1998). Like EREBP, *ZmERF1* expression increased in K0326Y QPM compared to W64A*o2* and W64A+ (Fig. 4g). Two from each phenotypic class of RILs (164 and 208, 175 and 217, and 39 and 194) showed significantly elevated

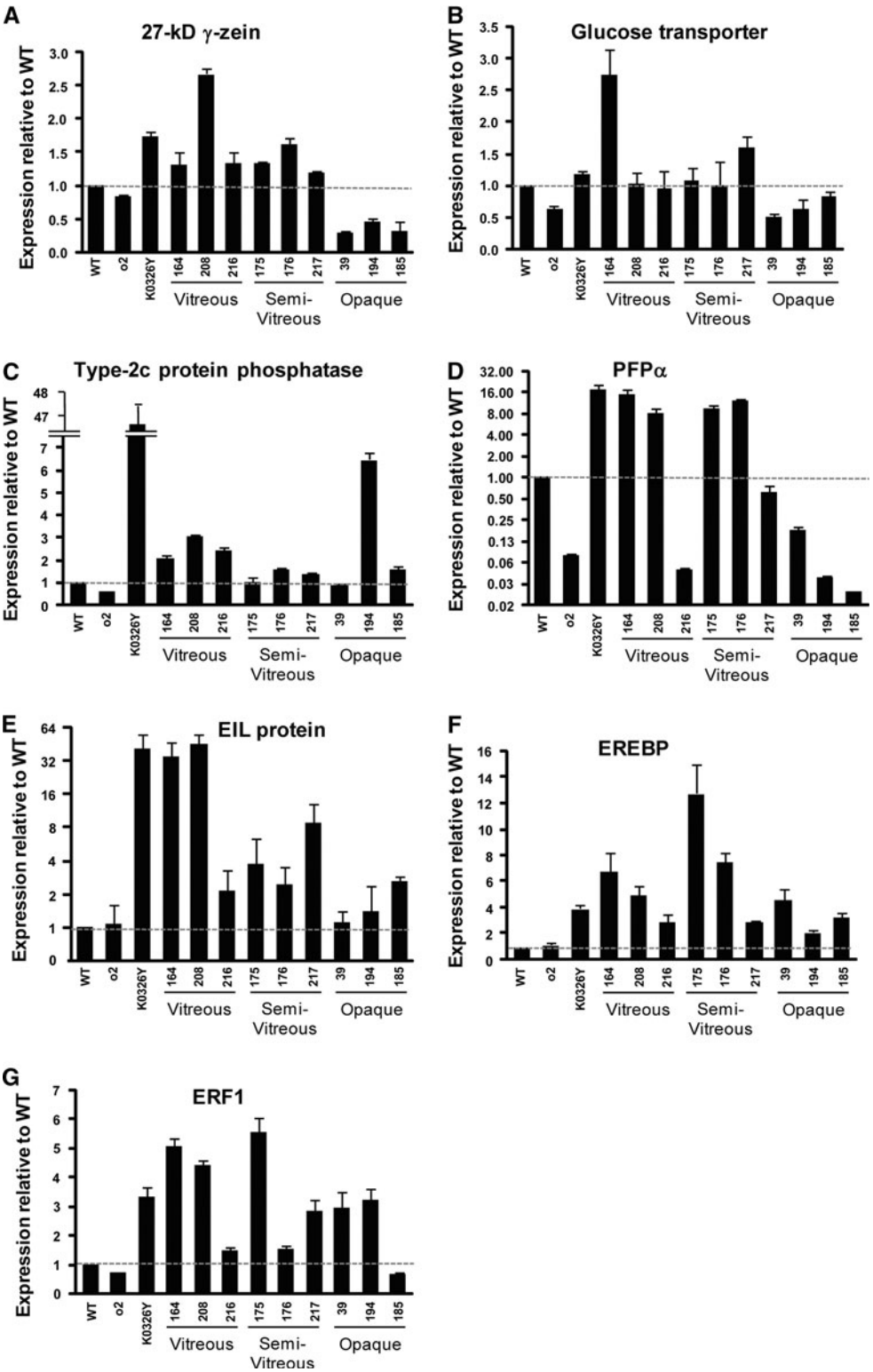

Fig. 4 Expression of *opaque2* modifier candidate genes in W64A+, W64A*o2* and K0326Y QPM and their associated RILs. *Y* axis values represent expression relative to W64A+ in **a**–**g** (set to one relative expression unit), which are shown on a $\log_2$ scale in **d** and **e** for comparison

ZmERF1 expression, like K0326Y QPM. Thus, while increased EIL expression correlated well with the vitreous phenotype of RILs, ethylene signaling as measured by the expression of two putative downstream transcription factors appeared to be increased in all the RILs examined, regardless of the phenotype. Consequently, components of the ethylene-signaling pathway could be generally involved in *o2* endosperm modification.

Discussion

In order to further characterize *Opm* loci in K0326Y QPM, we developed 314 RILs from an F2 ear of the cross with W64A*o2* (Holding et al. 2008). The RILs manifest a broad range of kernel phenotypes for vitreousness, density, and resistance to grinding (hardness), as well as a diverse combination of the QTLs found to influence *o2* endosperm modification (Table S1). The relationships between these kernel phenotypes and endosperm texture are poorly understood (Chandrashekar and Mazhar 1999; Fox and Manley 2009), but they are generally thought to describe this important agronomic trait (Fox and Manley 2009). Kernel texture is affected by the protein content of the seed and the conditions of the kernel during dry-down and desiccation (Kirlies and Stroshine 1990; Mestres and Matencio 1996). It also appears to be influenced by the structure of starch grains in the endosperm, which implies that carbohydrate synthesis could also be an important factor contributing to kernel texture (Gibbon et al. 2003). Based on the diversity of QTLs associated with kernel texture identified in previous studies of wild type (Séne et al. 2001) and modified *o2* maize (Lopes and Larkins 1995; Holding et al. 2008; Gutiérrez-Rojas et al. 2010), the genetic basis for kernel texture is complex and may differ among genetic backgrounds. Indeed, the genes responsible for texture in wild type kernels could be different from those important for *o2* endosperm modification.

Based on the BSA of true-breeding vitreous and opaque progeny from the K0326Y QPM by W64A*o2* cross, we identified QTLs affecting vitreousness on chromosomes 7 and 9. These same QTLs were confirmed in an analysis of individual F2 kernels of this cross, and were found to have major effects, i.e., contributing to 40% of the phenotypic variation. Analysis of the F2 population also identified QTLs on chromosomes 1, 5 and 10 that each accounted for 5–10% of the phenotypic variation. The RIL population described in the present study manifested the previously identified QTLs associated with vitreousness on chromosomes 1, 7, and 9, accounting for 56% of the phenotypic variation, which attests to the penetrance of these loci. The availability of larger quantities of seed made it possible to also phenotype the RILs for density and hardness. We observed a good correlation between vitreousness and density and between vitreousness and hardness. The correlation between vitreousness and density was supported by the QTL mapping data, as the SSR markers on chromosomes 7 and 9 that identified QTLs affecting vitreousness also identified QTLs associated with density. Hardness and density were not as highly correlated as the other two phenotypes, but these traits shared the same QTL on chromosome 7. These results are consistent with our previous studies that showed the QTL on chromosome 7 has a major effect on *o2* endosperm modification (Lopes and Larkins 1995; Holding et al. 2008). Considering the size of this QTL and the fact that it appears to influence multiple phenotypes, it is likely to contain several different genes affecting these traits.

Based on differential expression in a microarray hybridization experiment, we previously reported 16 genes that were differentially regulated in 18 DAP endosperm of K0326Y QPM relative to W64A*o2* (Holding et al. 2008). Although the higher level of expression of these genes was confirmed by real-time PCR, the oligonucleotide sequences used as primers were based on gene sequences in the B73 inbred. Because of the allele diversity in maize, it was important to confirm these results based on the nucleotide sequences of the genes in K0326Y QPM and W64A*o2*. Indeed, we found polymorphisms in several of these alleles that affected the primer sequences, resulting in misleading real-time PCR data. Of the 16 genes previously described, 10 were re-analyzed after design of primers common to the W64A*o2* and K0326Y QPM alleles, and six showed differential expression (Fig. 4).

In addition to examining the transcript levels of these differentially expressed genes in the parental inbreds, we also measured their expression in a small subset of the vitreous, semi-vitreous and opaque RILs (Fig. 3). These RILs were analyzed before the QTL mapping was completed, so not all combinations of the parental sources of QTLs are represented among them. Most of the QTLs in the opaque RILs came from the W64A*o2* parent, except for RIL 39, which has K0326Y QPM SSR markers flanking the locus on chromosome 1 (Table 3). The vitreous and semi-vitreous RILs have a diverse combination of QTLs from both parental inbreds. For example, RIL 164 has W64A*o2* markers flanking the QTL on chromosome 1, K0326Y QPM markers flanking the QTL on chromosome 7, and the QTL on chromosome 9 is flanked by an SSR marker from each parent. RIL 208 has K0326Y SSR markers flanking the QTLs on chromosomes 1 and 9, and a combination of W64A*o2* and K0326Y QPM SSR markers flanking the QTL on chromosome 7. Because of the size of the chromosomal regions defined by the SSRs flanking the QTLs, there are clearly many genes within each of these regions. Without additional knowledge of the precise sites of recombination of the parental genomes, it is premature to speculate on the allelic composition of the QTLs in these RILs. Furthermore, the map position of the genes up-regulated in K0326Y QPM and the QTLs could simply be coincidental.

One of the prominent biochemical features of QPM is a substantial increase in 27-kDa γ-zein protein (Wallace et al. 1990). Whereas *o2* mutants have a consistently reduced level of 27-kDa γ-zein protein compared with wild type, QPMs show higher expression of 27-kDa γ-zein both

at the transcript and protein levels (e.g. Figs. 1, 4a) (Geetha et al. 1991; Lopes and Larkins 1991; Moro et al. 1995). Recently, Wu et al. (2010) demonstrated that increased synthesis of 27-kDa γ-zein protein appears to be essential for the vitreous phenotype of one modified *o2* genotype, which could at least partially explain the QTL affecting vitreousness on chromosome 7. The mechanism by which large amounts of 27-kDa γ-zein create a vitreous endosperm in modified *o2* mutants is unclear, but it might be explained by the increased number of small protein bodies compared with starchy *o2* mutants (Holding and Larkins 2006).

The inter-conversion of fructose-1-phosphate and fructose-1,6-bisphosphate, a key rate-limiting step in glycolysis, is primarily catalyzed by an ATP-dependent phosphofructokinase in the forward direction and by fructose-1,6-bisphosphate phosphatase in the reverse direction. In plants, this reaction is also catalyzed by pyrophosphate-dependent fructose-6-phosphate 1-phosphotransferase, also known as pyrophosphate-dependent phosphofructokinase or PFP. PFP is a reversible enzyme, using pyrophosphate (PPi) in the forward direction and liberating PPi in the reverse direction. PFP usually exists as a hetero-tetramer with two regulatory α subunits and two catalytic β subunits, although a variety of different compositions both between and within species have been reported (Wong et al. 1990; Botha and Botha 1991). Our results show that the expression of a gene encoding the regulatory α subunit is dramatically increased in K0326Y QPM in relation to W64A+, and furthermore that its expression is substantially reduced in W64A*o2* compared to W64A+.

The function of PFP in plants is not clear, although several roles related to the modulation of respiration have been proposed (Plaxton and Podesta 2006). PPi is an alternative energy donor to ATP in plant cells and most evidence suggests that PFP functions in the glycolytic direction as an adaptive enzyme that enables plants to survive certain stresses by inducing PPi-dependent bypasses in order to conserve diminished ATP reserves (Plaxton and Podesta 2006).

The *O2* gene encodes a bZIP transcription factor that regulates α-zein gene expression, as well as that of other genes. The pleiotropic nature of the *o2* mutation was illustrated by a greater than threefold differential expression of more than 120 genes compared to wild type (Hunter et al. 2002). Many of the up-regulated genes were annotated as having functions in various cellular stress responses and protein turnover. These processes require substantial amounts of ATP. This implies that during *o2* kernel maturation ATP pools become depleted, which could be alleviated by increased PFP activity in K0326Y QPM. We recently completed a deep transcriptome sequencing experiment, which confirmed that many stress-related genes are up-regulated in W64A*o2*; furthermore, these genes are returned to normal expression levels in K0326Y QPM, suggesting that *o2* modifiers may function by alleviating stress responses (D. Holding, unpublished data). Nevertheless, how alleviating these stresses relates to the formation of vitreous endosperm during kernel maturation is unknown.

We found a twofold reduction in W64A*o2* for the expression of a gene encoding a glucose transporter. The W64A+ level of expression was observed in the vitreous and semi-vitreous RILs, while expression in the opaque RILs resembled that of W64A*o2*. This gene belongs to the Major Facilitator Superfamily (Pao et al. 1998). Due to the diverse nature of these transporters, without further work to characterize and localize this protein, we can only speculate on the functional significance of this difference in expression. In addition to serving as a substrate for starch biosynthesis, glucose is also a signaling molecule, especially as an antagonist to ethylene (Smeekens 2000). Glucose has been shown to promote the degradation of the EIN3 protein, a transcription factor with a key positive role in ethylene signaling (Yanagisawa et al. 2003).

We determined a 3.3-fold increase in expression of a maize *EIN3*-like gene (*EIL*) in K0326Y QPM compared to W64A*o2* (Holding et al. 2008). However, using primers in regions known to be 100% identical in W64A*o2* and K0326Y QPM, we found a 40-fold higher expression in K0326Y QPM, and the expression of this gene was significantly up-regulated in both the vitreous and semi-vitreous RILs compared to the opaque RILs (Fig. 4f). If increased EIL expression plays a role in the formation of vitreous endosperm, it is interesting that this may also be accompanied by altered glucose partitioning. A decrease in cytosolic glucose (increased in the plastid) would be consistent with reduced glucose-mediated EIL degradation. Alternatively, increased glucose transport into the cytoplasm or the nucleus could be a response to an EIL increase, serving to keep its activity as a transcription factor in check. An increase in ethylene signaling in K0326Y QPM is supported by the increased expression of transcription factors that are downstream targets of *EIL*. These are an ethylene responsive element binding protein (EREBP) in the same BAC contig as the 27-kDa γ-zein gene on chromosome 7 (Holding et al. 2008) and the putative ortholog of the Arabidopsis ERF1 gene (Solano et al. 1998). Unlike *EIL*, for which expression correlates quite well with vitreousness in the RILs, expression of the downstream effector genes is generally increased in most of the RILs, regardless of their phenotype. This suggests that other factors, such as *EIL* stability, are also involved in the expression of downstream genes. Ethylene signaling is involved in endosperm programmed cell death (PCD) (Young et al. 1997), which begins in the center of the endosperm and spreads up to the aleurone layer at kernel

maturity. It is counterintuitive to believe that increased PCD favors the formation of vitreous endosperm in QPM, but it is possible that metabolic stresses associated with the *o2* mutation are ameliorated by *o2* modifiers, leading to a normal endosperm phenotype.

The results of these experiments suggest that several different genes and metabolic pathways can contribute to the formation of vitreous endosperm in QPM. Whether the candidate genes we have identified are necessary or sufficient to attain a vitreous phenotype is unclear. At this point in our investigation, it is difficult to assign importance to a particular gene or pathway, although a case can be made for the 27-kDa γ-zein protein (Wu et al. 2010).

While many questions remain to be answered regarding the high expression level of genes differentially expressed in K0326Y QPM relative to W64A*o2*, the functions of these genes provide clues to metabolic pathways that appear to be altered in modified *o2* backgrounds. We have identified 40 RILs that contain single or combinations of *Omp* QTLs on chromosomes 1, 7, and 9, and these will be used to evaluate the relationship between kernel phenotype and expression of the candidate genes associated with specific QTLs. We are also examining the expression of these genes in other QPM backgrounds and creating transgenic lines that overexpress these genes to determine the degree to which they singly, or in combination, influence the formation of a vitreous kernel phenotype in *o2* genotypes.

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