Genetic and molecular analysis of starch physicochemical properties and its relationship with endosperm modification in quality protein maize

Análisis genético y molecular de propiedades fisicoquímicas del almidón y su asociación con la modificación del endospermo en maíz de calidad proteínica

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ABSTRACT

Quality protein maize (QPM) was created converting the soft *opaque-2* endosperm into a vitreous phenotype, but the mechanisms involved in this modification are not completely understood. Recombinant inbred lines (RILs) derived from K0326Y QPM and W64A*o2* were used to identify quantitative trait loci (QTL) associated with starch physicochemical properties. Vitreousness-contrasting RILs were also used to evaluate the expression of starch biosynthesis genes. Mapping identified 5-6 QTLs for each trait that explained 66 % of the phenotypic variation. Three QTLs on bins 4.05, 5.04, and 9.03 were found close to the starch biosynthesis genes *Brittle-2* (*Bt2*), *Amylose extender-1* (*Ae1*), and *Waxy-1* (*Wx1*), respectively. The expression of *Wx1* was three-fold greater in K0326Y QPM than W64A*o2*, and eight-fold higher in vitreous than opaque RILs, which corresponded with the greater granule bound starch synthase I (GBSSI) accumulation and the higher amylose content observed in the vitreous lines. The increased synthesis of amylose may result in starch granules with more amorphous regions that favor their compaction. These results suggest that endosperm modification in QPM is associated with the synthesis of starch containing more amylose during kernel development, which may facilitate the packing of the starch granules resulting in the vitreous phenotype.

Keywords: Quality protein maize, endosperm modification, starch, gene expression.

RESUMEN

El maíz de calidad proteínica (QPM) fue creado convirtiendo el endospermo suave *opaco-2* en un fenotipo vítreo, pero los mecanismos involucrados en esta modificación no se conocen por completo. Se utilizaron líneas recombinantes puras (RIL) derivadas de las líneas K0326Y QPM y W64A*o2* para identificar *loci* de características cuantitativas (QTL) asociados con propiedades fisicoquímicas del almidón. También se usaron RILs contrastantes en vitrosidad para evaluar la expresión de genes de biosíntesis de almidón. El mapeo identificó 5 o 6 QTL para cada característica que explicaron en promedio el 66 % de la variación fenotípica. Tres de los QTLs en los bins 4.05, 5.04 y 9.03 se encontraron cerca de los genes de síntesis de almidón *Brittle 2* (*Bt2*), *Amylose extender 1* (*Ae1*), y *Waxy 1* (*Wx1*), respectivamente. La expresión de *Wx1* fue tres veces mayor en K0326Y QPM que en W64A*o2* y ocho veces mayor en líneas vítreas en comparación con las opacas, lo que correspondió con la mayor acumulación de la enzima almidón sintasa unida al gránulo I (GBSSI) y el mayor contenido de amilosa observado en las líneas vítreas. El incremento en la síntesis de amilosa podría resultar en gránulos de almidón con más regiones amorfas que favorecen su compactación. Estos resultados sugieren que la modificación del endospermo en QPM está asociada con la síntesis de almidón conteniendo más amilosa durante el desarrollo del grano, lo cual podría facilitar el empaquetamiento de los gránulos de almidón resultando en el fenotipo vítreo.

Palabras clave: Maíz de calidad proteínica, modificación del endospermo, almidón, expresión génica.

INTRODUCTION

Maize (*Zea mays* L.) is one of the most important staple foods in developing countries, but it has a poor protein quality due to the deficiency of lysine (Lys) and tryptophan (Trp). The maize *opaque2* mutation typically doubles the Lys and Trp contents of wild-type kernels (Mertz *et al*., 1964). However, the agronomic problems associated with the soft endosperm of this mutant limited its use. Plant breeders developed hard-kernel *o2* genotypes known as Quality Protein Maize (QPM) (Villegas *et al*., 1992), but the mechanism by which the soft endosperm of *o2* becomes hard and vitreous in QPM is not entirely understood. Some studies have demonstrated an essential role for the 27-kDa γ-zein in endosperm modification (Wu *et al*., 2010; Liu *et al*., 2016); the enhanced accumulation of 27-kDa γ-zein favors the formation of protein bodies and their compaction between starch granules. Gibbon *et al*. (2003) proposed a complementary mechanism where alterations in starch structure facilitate that granules associate with one another, and the spaces between them are filled in the vitreous areas of the kernel.

Genetic analysis with recombinant inbred lines (RILs) derived from a cross between W64A*o2* and K0326Y QPM

identified three quantitative trait loci (QTL) in bins 1.06, 7.02, and 9.03 associated with endosperm modification (Holding *et al.*, 2011). The locus in bin 7.02 was linked to the 27-kDa γ-zein, whereas that in bin 9.03 was close to starch biosynthesis genes. Salazar-Salas *et al*. (2014) evaluated the effect of each QTL on protein synthesis and starch physicochemical properties in the mature endosperm. They found that the vitreous phenotype of QPM is associated with the synthesis of less crystalline starch containing more amylose and short-intermediate amylopectin branch chains, which may favor starch granules packing. However, knowledge about the genetic and molecular basis of these changes in starch composition is scarce. This study aimed to identify QTL associated with starch physicochemical properties, and to evaluate the expression of starch biosynthesis genes and its relationship with starch composition in developing endosperm of maize lines contrasting in vitreousness and the genotype of QTL flanking markers.

MATERIALS AND METHODS
Plant materials
Maize inbred lines K0326Y QPM (vitreous) and W64A*o2* (opaque) and 103 recombinant inbred lines (RIL) derived from their cross were used. They were grown at the Faculty of Agronomy field station in Culiacan, Sinaloa, Mexico (24°37′29″ N, 107°26′37″ W), in 2015. Kernels were degermed and ground into a fine powder. Based on QTLs previously identified for endosperm modification (Holding *et al.*, 2011), six RILs contrasting in vitreousness and the genotype of flanking markers were selected. Three vitreous RILs were homozygous for the K0326Y QPM allele and three opaque RILs were homozygous for the W64A*o2* allele. Developing kernels were obtained from the selected RILs 30 days after pollination (DAP), frozen in liquid nitrogen, and stored at - 80 °C until use.

Starch composition
The K-TSTA and K-AMYL assays (Megazyme, Wicklow, Ireland) were used to determine total starch and amylose contents from developing and mature endosperm samples, respectively. Amylopectin content was obtained by difference.

Starch swelling power
It was determined according to Konik-Rose *et al*. (2001). About 40 mg of flour were mixed with 1 mL of water, vortexed, and then incubated for 30 min at 92.5 °C. The mixture was cooled and centrifuged (17,000 x g, 10 min). The swelling power was calculated by dividing the weight of the residue by the initial weight of flour.

Gelatinization enthalpy
Flour samples (2 mg) were mixed with deionized water (7 µL) and sealed in hermetic pans for analysis with a differential scanning calorimeter (DSC-2920, TA Instruments, New Castle, USA) calibrated with indium; an empty pan was used as a reference. The heating rate was 10°C/min over the temperature range (30-120 °C). Gelatinization enthalpy (ΔH_G) was calculated with TA Universal Analysis 2000 software (TA Instruments).

Genetic mapping
MapManager QTxb20 software (Manly *et al.*, 2001) and SSR linkage groups previously reported for the RIL population (Holding *et al.*, 2011) were used to identify QTL associated with starch composition, starch swelling, and gelatinization enthalpy by composite interval mapping (CIM) (Zeng, 1994). CIM was conducted with an additive regression model, and permutation tests (1000 shuffles) on individual chromosomes were performed to establish the significant threshold value of the likelihood ratio statistic (LRS) (Churchill and Doerge, 1994).

RNA isolation and cDNA synthesis
Total RNA was isolated from developing endosperm (30 DAP) according to Holding *et al*. (2008). Endosperms were ground in 200 µL of RNA extraction buffer [50 mmol/L Tris-HCl, pH 8, 150 mmol/L LiCl, 5 mmol/L EDTA, pH 8, 10 g/L SDS], added with 200 µL of phenol/chloroform (1:1) pH 8, mixed and placed 5 min on ice. The aqueous phase was separated (13,000 x *g*, 10 min, 4 °C). Starch was removed using 1 mL trizol reagent (Life Technologies), followed by the addition of 200 µL of phenol-chloroform (1:1), and then centrifuged (10,000 x *g*, 10 min, 4 °C). Nucleic acids were precipitated with one volume of isopropanol, centrifuged (10,000 x *g*, 10 min, 4 °C), the pellet washed with 70 % ethanol and resuspended in 50 µL DEPC water. The obtained RNA was treated with DNase using the DNase I Kit (Sigma-Aldrich, St. Louis, USA). cDNA was synthesized from 2 µg of RNA using the Go Script TM reverse transcription system kit (Promega, Madison, USA).

Gene expression analysis
RT-qPCR was used to determine the 27-kDa γ-zein and starch biosynthesis genes transcript levels. The cDNA (75 ng) was amplified using the *Power* SYBR® Green PCR Master Mix (Applied Biosystems, UK). The PCR primers (Table 1) were designed using the Primer-BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast). A real-time system StepOnePlusTM (Applied Biosystems, Carlsbad, USA) was used under the following conditions: 95 °C for 10 min, then 40 cycles at 95 °C (15 s) and 60 °C (1 min) with a ramp rate of 2.2 °C/s. The gene encoding retinoblastoma-related protein 1 (*RRB1*) was used as a control, and the expression values were calculated relative to the parental line K0326Y QPM (vitreous), which is the donor of the modifier genes that convert the soft endosperm of W64A*o2* to a vitreous phenotype in the RILs. The relative expression was calculated according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) using the following equation: $2^{[(WX-WC) - (GX-GC)]}$, where X= gene of interest, C= control gene (RRB1), W=average cycle threshold (C_t) of K0326Y QPM, and G= average C_t of the other genotypes.

Tabla 1. Iniciadores específicos usados para RT-qPCR.
Table 1. Gene-specific primers used for RT-qPCR.

Gene		Primer	Amplicon size (bp)
γ-*zein*	Fw	GTATAAGTCCTAAAGTGGTGAGGAACA[1]	240
	Rv	GAGCTGTAAGCATGTTATCAGATGAG[1]	
Bt2	Fw	ATTACCGTTGCTGCCCTACC[2]	115
	Rv	ACTGCTCTCCTTTCGGTTTCTC[2]	
Wx1	Fw	GAGGACGTCGTGTTCGTCTG[3]	245
	Rv	CAGTTGATCTTCCGGCCTTC[3]	
Ae1	Fw	CCGACGCTGGACTATTTGGT[3]	165
	Rv	GCAACGAGTACCCCGCTATC[3]	
Zpu1	Fw	GCTTCAGTTGCATCCAGTGC[3]	250
	Rv	TGGGCAACGTCTTATTCAATC[3]	
RRB1	Fw	GCTGTTTCTGGTTATGTCTGTCCT[3]	138
	Rv	CTTTTGAGTACTTCTGTGCCTGAC[3]	

γ-*zein:* 27kDa γ-*zein*; *Bt2: Brittle-2* (ADP-glucose pyrophosphorylase, small subunit); *Wx1: Waxy-1* (Granule-bound starch synthase 1); *Ae1: Amylose extender 1* (Starch-branching enzyme); *Zpu1*: Pullulanase-type starch debranching enzyme 1; *RRB1*: Retinoblastoma-related protein 1; Fw, Forward; Rv, Reverse. [1]Holding *et al*. (2011); [2]Huang *et al*. (2011); [3]Jia *et al*. (2013).

Zein Extraction, Quantification, and SDS-PAGE Analysis

Developing endosperms were lyophilized and ground into a fine powder to obtain the zein fractions as described by Wallace *et al*. (1990). Total protein from 50 mg of endosperm flour was extracted overnight with 1 mL of borate buffer (12.5 mmol/L $Na_2B_4O_7$-$10H_2O$, 10 g/L SDS, 20 mL/L 2-mercaptoethanol, pH 10) at 37 °C. The extract was adjusted with ethanol (700 mL/L) to precipitate the non-zein fraction. Zein protein extracts (equivalent to 15 mg of flour) were vacuum-dried and resuspended in 100 µL of deionized water. Five microliters were separated in 0.15 mg/mL polyacrylamide gels (SDS-PAGE) and stained with Coomassie Blue R250. The Quantity One software (BioRad, Hercules, CA) was used to quantify by densitometry the protein bands.

Isolation of starch granules, extraction and identification of starch granule-associated proteins (SGAP)

Starch granules were isolated from developing (30 DAP) kernels, according to Grimaud *et al*. (2008). Kernels were soaked in 3 g/L sodium metabisulfite and 850 mL/L lactic acid (pH 3.8) for 48 h at 50 °C, and then the germ and pericarp were manually dissected. Endosperms were ground with a mortar and pestle in starch extraction buffer [50 mmol/L Tris-HCl, pH 7, 100 mL/L glycerol, 10 mmol/L EDTA, 1.25 mmol/L DTT] at 4 °C, passed through a 100 µm filter and centrifuged at 13,000 x g for 15 min. Starch was washed five times with cold extraction buffer, 950 mL/L ethanol, and acetone and then dried for 12 h. For SGAP extraction, about 150 mg of starch were washed at 4 °C with water, gelatinization buffer [62.5 mmol/L Tris-HCl, pH 6.8, 20 g/L SDS, 50 mL/L β-mercaptoethanol], and 950 mL/L ethanol to remove superficial proteins. Then, starch samples were incubated with gelatinization buffer for 15 min in a boiling water bath to release SGAP. The paste formed

was frozen for 1 h, thawed for 20 min at 40 °C in a water bath and then centrifuged (13,000 x g, 30 min). The supernatant was collected and mixed with an equal volume of 300 g/L TCA/Acetone for at least 2 h to precipitate proteins. After centrifugation for 15 min at 4 °C, the SGAP pellet was washed three times with cold acetone and dried overnight at room temperature. SGAP were resuspended in thiourea buffer [7 mol/L Urea, 2 mol/L Thiourea, 40 g/L CHAPS, 20 mmol/L DTT, 2 mL/LTriton-X100]. Protein extracts equivalent to 8 and 21 mg of starch were used for granule-bound starch synthase and low abundant SGAP, respectively; they were separated by SDS-PAGE using a 0.10 mg/mL polyacrylamide gel and identified by mass spectrometry according to Salazar-Salas *et al*. (2014).

Statistical analysis

Data correspond to the mean of three biological replicates with three technical replicates. It was analyzed using one-way ANOVA, and the Fisher test ($p < 0.05$) was used for mean comparisons. These analyses were performed using the software STATGRAPHIC plus version 5.1 (Statistical Graphics Corporation™, Maryland, USA).

RESULTS AND DISCUSSION
Identification of QTL associated with starch properties

The phenotypic variation of starch, amylose, amylopectin content, swelling power, and gelatinization enthalpy was determined in 103 RIL derived from K0326Y QPM and W64A*o2* (Table 2). A continuous variation was observed in the RIL population for every trait studied, and the extreme values were outside of those from the parental lines, indicating transgressive segregation. Composite interval mapping identified 26 QTL, which explained 66% of the total variation for the starch properties (Table 3). For total starch, five loci were identified on chromosomes 1 (qST-1), 4 (qST-4), 5 (qST-5), 7 (qST-7), and 10 (qST-10); the alleles contributing to this trait were mainly from K0326Y QPM, except for two loci contributed by the W64A*o2* alleles. Similar chromosomal locations associated with QTLs for starch content have been reported in different maize germplasm (Séne *et al*., 2001; Zhang *et al*., 2008; Dong *et al*., 2015).

Five QTLs were identified for amylose content (Table 3), and the favorable alleles came from K0326Y QPM. These loci were also identified for amylopectin content but in this case, the positive additive effect corresponded to alleles from W64A*o2* (high amylopectin). The major QTLs for these traits were in bins 3.05-3.06 (qAM-3, qAP-3), 7.02 (qAM-7, qAP-7), and 9.03-9.04 (qAM-9, qAP-9), explaining 47 % of the observed variation. The loci on chromosomes 1, 7, and 9 coincided with the QTL reported by Holding *et al*. (2011) for traits associated with *o2* endosperm modification (vitreousness, density, and hardness) in the same RILs. A significant correlation was observed between amylose content and vitreousness in the RIL population (r = 0.52, p < 0.01).

Five QTL were identified for starch swelling power (SP) on chromosomes 1 (qSP-1), 2 (qSP-2), 5 (qSP-5), 7 (qSP7), and 9 (qSP-9) (Table 3), highlighting qSP-9 that explains 23 %

Tabla 2. Variabilidad de propiedades fisicoquímicas de almidón en endospermo maduro de K0326Y QPM, W64A*o2* y líneas recombinantes puras (RIL) derivadas de su cruza.
Table 2. Variability of starch physicochemical properties in mature endosperm of K0326Y QPM, W64A*o2* and recombinant inbred lines (RIL) derived from their cross.

Property	K0326Y QPM	W64A*o2*	RILs
Total starch (mg /100 mg flour)	74.00 ± 0.80[a]	75.00 ± 0.80[a]	63.21 - 76.79
Amylose (mg /100 mg starch)	25.54 ± 1.06[a]	21.79 ± 0.49[b]	15.56 - 32.29
Amylopectin (mg /100 mg starch)	74.46 ±1.06[b]	78.21 ± 0.49[a]	67.71 - 89.91
Swelling power (g /g flour)	7.19 ± 0.01[b]	9.22 ± 0.08[a]	5.89 - 10.09
Gelatinization enthalpy (J/g)	10.35 ± 0.13[b]	11.54 ± 0.52[a]	8.14 - 13.41

Different superscript letters between the parental lines K0326Y QPM and W64A*o2* indicate significant differences based on the Fisher test (P<0.05).

of the variation. The qSP-1, qSP-7, and qSP-9 loci coincided with those identified for amylose (Table 3) and vitreousness (Holding *et al*., 2011). Alleles from W64A*o2 (soft endosperm)* contributed to the variation, which corresponds with the high SP and low amylose values of this parental line and the inverse correlation observed for these traits in the RILs (r = -0.413, p < 0.01). SP is frequently used as an indirect measure of water uptake during starch gelatinization (Konik-Rose *et al*., 2001) and is influenced by the granule composition. The asymmetric distribution of amylopectin molecules favors the entry of water and thereby the swelling power, whereas amylose can form amylose-lipids complexes that inhibit swelling (Tester and Morrison, 1990).

Four of the six QTL identified for gelatinization enthalpy were coincident with those for SP on chromosomes 1 (qGE-1.2), 2 (qGE-2), 7 (qGE-7), and 9 (qGE-9), which together explained 51 % of the ΔHG variation (Table 3). Similar to SP, loci on chromosomes 7 and 9 were coincident with those

Tabla 3. Características de QTLs asociados con propiedades del almidón.
Table 3. Characteristics of QTLs associated with starch properties.

Trait	QTL	Bin	Left-Right markers	LRS	Variance	**Effect***
Total starch	qST-1	1.07-1.08	Umc1278-Dupssr12	12.2	11	-0.96
	qST-4	4.04-4.05	Mmc0471-Bnlg1937	13.8	14	1.02
	qST-5	5.03-5.04	Umc1056-Bnlg2323	12.7	12	-0.96
	qST-7	7.04	Umc1029-Umc1944	12.2	11	0.86
	qST-10	10.04-10.06	Umc1053-Umc2190	12.7	11	-0.99
Amylose	qAM-1	1.05-1.06	Umc1076-Umc1335	11.3	9	-1.14
	qAM-3	3.05-3.06	Umc1167-Bnlg197	15.6	14	-1.32
	qAM-5	5.04-5.05	Bnlg2323-Mmc0081	13.0	12	-1.29
	qAM-7	7.02	Umc1036-γ zein	19.6	19	-1.65
	qAM-9	9.03-9.04	Bnlg127-Umc1771	16.4	14	-1.27
Amylopectin	qAP-1	1.05-1.06	Umc1076-Umc1335	11.3	9	1.14
	qAP-3	3.05-3.06	Umc1167-Bnlg197	15.6	14	1.32
	qAP-5	5.04-5.05	Bnlg2323-Mmc0081	13.0	12	1.29
	qAP-7	7.02	Umc1036-γ zein	19.6	19	1.65
	qAP-9	9.03-9.04	Bnlg127-Umc1771	16.4	14	1.27
SP	qSP-1	1.05-1.06	Umc1076-Umc1335	12.8	12	0.30
	qSP-2	2.08-2.09	Umc1516-Umc1551	11.7	10	0.28
	qSP-5	5.05	Umc1264-Bnlg278	11.9	10	0.31
	qSP-7	7.02	Umc1036-γ zein	23.7	12	0.39
	qSP-9	9.03	Wx1-Bnlg127	28.5	23	0.43
ΔHG	qGE-1.1	1.03	Bnlg176-Bnlg439	19.3	16	0.42
	qGE-1.2	1.05-1.06	Umc1076-Umc1335	10.7	9	0.41
	qGE-2	2.08-2.09	Umc1516-Umc1551	13.6	12	0.41
	qGE-5	5.08-5.09	Umc1225-Umc1153	11.0	10	0.36
	qGE-7	7.02	Umc1036-γ zein	11.8	11	0.40
	qGE-9	9.03	Wx1-Bnlg127	14.9	13	0.47

LRS, likelihood ratio statistic. *The additive effect is negative for the K0326Y-QPM allele and positive for the W64A*o2* allele. SP, Swelling power. ΔHG, Gelatinization enthalpy.

previously reported for amylose (Table 3) and vitreousness (Holding *et al*., 2011); the W64A*o2* alleles contributed to the ΔHG variation, which corresponded with the higher gelatinization enthalpy observed for this parental line (Table 2). Gelatinization enthalpy has been used as a measure of starch crystallinity, which is related to the content of amylopectin (Matveev *et al*., 2001).

Candidate genes for the QTLs were proposed based on their proximity to flanking markers in the Maize Genome Database (https://www.maizegdb.org); co-locations between candidate genes and the QTLs were considered significant when the distances were lower than 10-20 cM (Séne *et al*., 2001). The flanking markers of QTLs for starch content and composition on bins 4.05 (Bnlg1937) and 5.04 (Bnlg2323) were found near *Brittle-2* (*Bt2*) and *Amylose extender-1* (*Ae1*) genes, respectively. *Bt2* encodes for the small subunit of ADP glucose pyrophosphorylase and *Ae1* for starch branching enzyme IIb (SBEII). The QTL on bin 9.03 (Wx1-Bnlg127) corresponds to the waxy gene (*Wx1*), which encodes for the GBSSI enzyme, responsible for the synthesis of amylose in the starch granule (Denyer *et al*., 2001).

The QTL on chromosome 7 (Umc1036-γ zein) was located in a locus corresponding to the 27-kDa γ-zein, which was previously reported for vitreousness, hardness, and density in the same RIL population (Holding *et al*., 2011). Transcription factors that regulate zein genes also appear to control starch synthesis (Zhang *et al*., 2016). A QTL on chromosome 1 was observed for all traits, but its effect is unclear since no candidate genes affecting starch properties were identified close to this locus; however, the flanking marker in bin 1.05 (Umc1076) is close to a gene for 19 kDa α-zein (*az19D1*). Salazar-Salas *et al*. (2014) reported higher levels of 19 kDa α-zein in vitreous than opaque lines and a highly significant correlation (r = 0.75, p < 0.01) between the

contents of this protein and those of 27 kDa γ-zein in the RIL population derived from K0326Y QPM and W64A*o2*. Both proteins may be contributing to protein bodies formation and the vitreous phenotype.

Starch composition in developing endosperm of QPM lines contrasting in vitreousness

The starch composition was determined in developing endosperms (30 DAP) of K0326Y QPM, W64A*o2,* and six RIL contrasting in kernel vitreousness (Table 4) to investigate its relationship with the expression of starch biosynthesis genes. The starch composition of mature endosperms (50 DAP) was included as a reference. At 30 DAP, total starch ranged from 59.1 to 64.6 %; these values were lower than the 88.9 % reported by Li *et al*. (2007) in B73 maize at the same developmental stage, which could be attributed to differences in genotype, growing conditions and method used for starch isolation. No significant differences were found in total starch content between the parental lines K0326Y QPM and W64A*o2*. Pereira *et al*. (2008) reported a similar behavior between an *opaque-2* and a QPM line at 30 DAP. The starch content increased to an average of 75 % in mature endosperms (Table 4).

The amylose content of developing endosperm from K0326Y QPM (20.0 %) was significantly higher (p ≤ 0.05) than that of W64A*o2* (17.3 %), which corresponded with the higher amylose content observed in the vitreous RIL with respect to the opaque lines (Table 4). The same behavior was observed in the mature endosperm. These results agree with those of Dombrink-Kurtzman and Knutson (1997) and Gayral *et al*. (2015), who observed higher amylose levels in starch from the vitreous region of maize endosperm with respect to starch from the floury area.

Tabla 4. Composición de almidón en endospermo en desarrollo (30 DDP) y maduro (50 DDP) de K0326Y QPM, W64A*o2*, y líneas recombinantes puras seleccionadas contrastantes en el genotipo de marcadores que flanquean loci asociados con la modificación del endospermo en QPM.
Table 4. Starch composition in developing (30 DAP) and mature (50 DAP) endosperms of K0326Y QPM, W64A*o2*, and selected recombinant inbred lines (RILs) contrasting in the genotype of markers flanking loci associated with endosperm modification in QPM.

Maize line		Starch*		Amylose**		Amylopectin**	
		50 DAP	30 DAP	50 DAP	30 DAP	50 DAP	30 DAP
K0326Y-QPM	V	59.4 ± 1.5[d]	74.0 ± 0.8[c]	20.0 ± 0.7[a]	25.5 ± 1.1[a]	80.0 ± 0.7[c]	74.5 ± 1.1[e]
W64Ao2	O	59.6 ± 1.1[cd]	75.0 ± 0.8[bc]	17.2 ± 0.5[b]	21.8 ± 0.5[b]	82.8 ± 0.5[b]	78.2 ± 0.5[d]
33	V	61.5 ± 1.4[bc]	77.6 ± 1.2[a]	16.5 ± 0.4[b]	21.7 ± 0.4[bc]	83.5 ± 0.4[b]	78.3 ± 0.4[cd]
338	V	61.0 ± 1.4[bcd]	75.8 ± 1.7[b]	16.6 ± 0.7[b]	22.0 ± 0.3[b]	83.4 ± 0.7[b]	78.0 ± 0.3[d]
346	V	62.3 ± 1.3[b]	75.9 ± 0.6[ab]	17.1 ± 0.4[b]	21.9 ± 0.5[b]	82.9 ± 0.4[b]	78.0 ± 0.5[d]
55	O	64.6 ± 0.7[a]	75.7 ± 0.7[b]	14.2 ± 0.4[c]	19.9 ± 0.7[de]	85.8 ± 0.4[a]	80.1 ± 0.7[ab]
73	O	62.5 ± 1.3[ab]	74.9 ± 0.6[bc]	14.6 ± 0.7[c]	19.5 ± 0.4[e]	85.3 ± 0.7[a]	80.5 ± 0.4[a]
185	O	59.5 ± 1.1[d]	76.1 ± 0.6[ab]	16.8 ± 0.1[b]	20.7 ± 0.5[cd]	83.2 ± 0.1[b]	79.3 ± 0.5[bc]

DAP, Days after pollination; V, vitreous; O, opaque; * Total starch expressed as mg per 100 mg of endosperm flour; ** Amylose and amylopectin expressed as mg per 100 mg of starch. For each property and developmental stage different superscript letters in the same column indicate significant differences between the maize lines based on the Fisher test (P < 0.5).

Expression analysis of QTL candidate genes

The molecular basis of QTLs associated with starch properties was investigated by measuring the expression of a 27-kDa γ-zein gene (Fig. 1) and four genes related to starch biosynthesis (*Bt2*, *Wx1*, *Ae1,* and *Zpu1*) (Fig. 2) in the parental lines and six RILs contrasting in vitreousness and the genotype of flanking markers. A late middle stage (30 DAP) of seed development was chosen because the endosperm cells have high levels of gene expression and large amounts of starch and storage proteins that may be associated with

the vitreous phenotype in mature endosperm. The relative expression of the 27-kDa γ-zein gene was significantly higher in K0326Y QPM and the vitreous RIL with respect to W64A*o2* and the opaque lines (Fig. 1A). Holding *et al*. (2011) also observed a higher expression of this gene in vitreous RILs after 18 DAP, indicating very low levels of transcripts for opaque samples as observed in the present study. This expression pattern was confirmed at the protein level in developing (30 DAP) endosperms (Fig. 1B); the mean value of 27-kDa γ-zein was 21.8 % higher in vitreous than opaque RILs. Sethi *et al*.
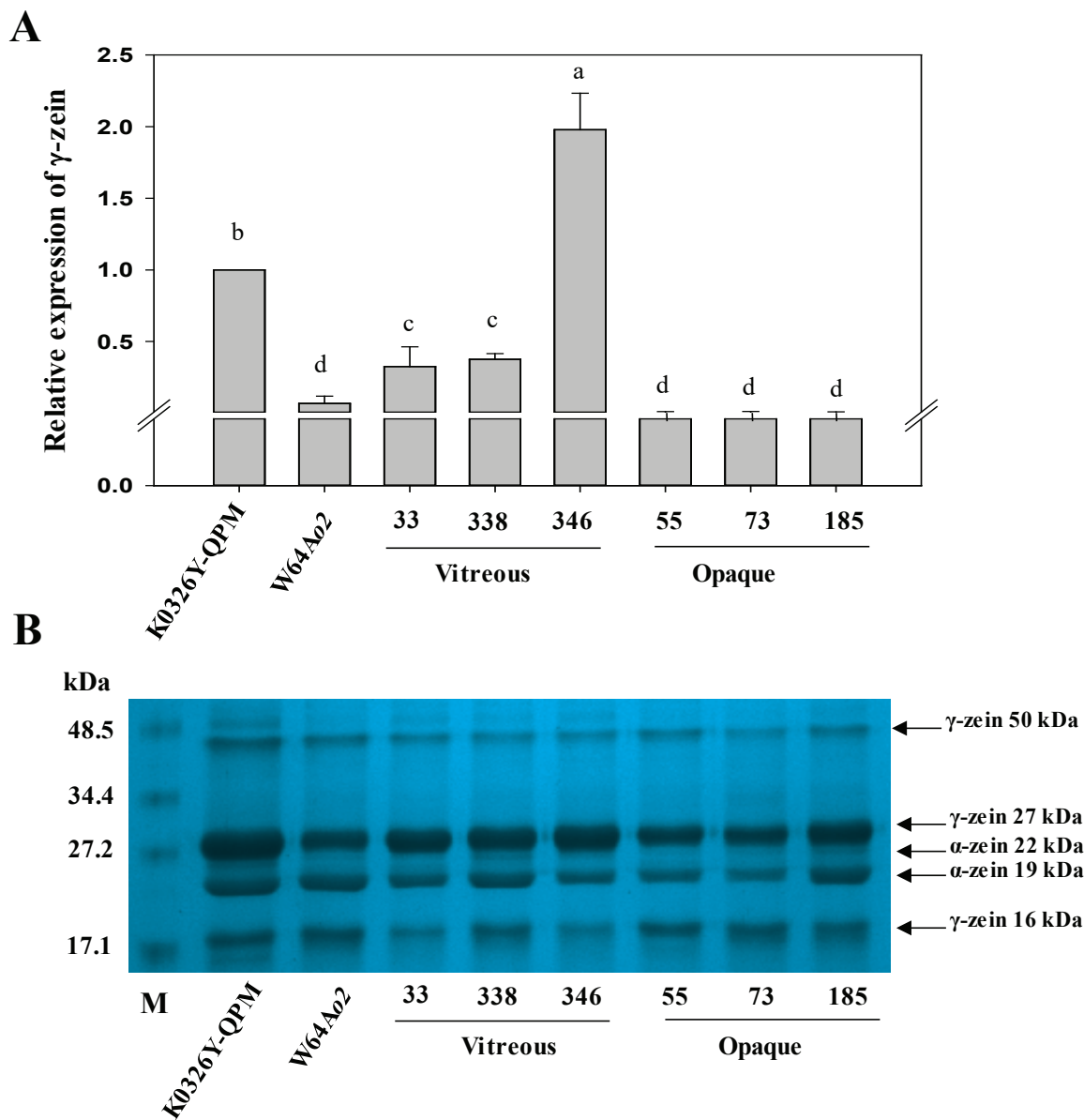

Figura 1. Expresión relativa del gen de γ–zeína y perfil de zeínas en endospermo en desarrollo (30 DDP) de líneas contrastantes en vitrosidad y el genotipo de marcadores que flanquean QTLs para esta característica. (A) Expresión relativa del gen de γ–zeína de 27 kDa. Letras diferentes indican diferencias significativas entre las líneas de maíz basado en la prueba de Fisher (P<0.05). (B) Separación por SDS-PAGE de zeínas de endospermo en desarrollo (30 DDP) (B). Los marcadores de peso molecular (kDa) se muestran en la izquierda y en la derecha se indican las principales sub-fracciones de zeínas.

Figure 1. Relative expression of γ–zein gene and zein profiles in developing endosperm (30 DAP) of lines contrasting in vitreousness and the genotype of QTLs-flanking markers for this trait. (A) Relative expression of the 27-kDa γ–zein gene. Different letters indicate significant differences between the maize lines based on the Fisher test (P<0.05). (B) SDS-PAGE separation of zein proteins. Molecular weight (kDa) standards are shown on the left, and the main zein sub-fractions are indicated on the right.
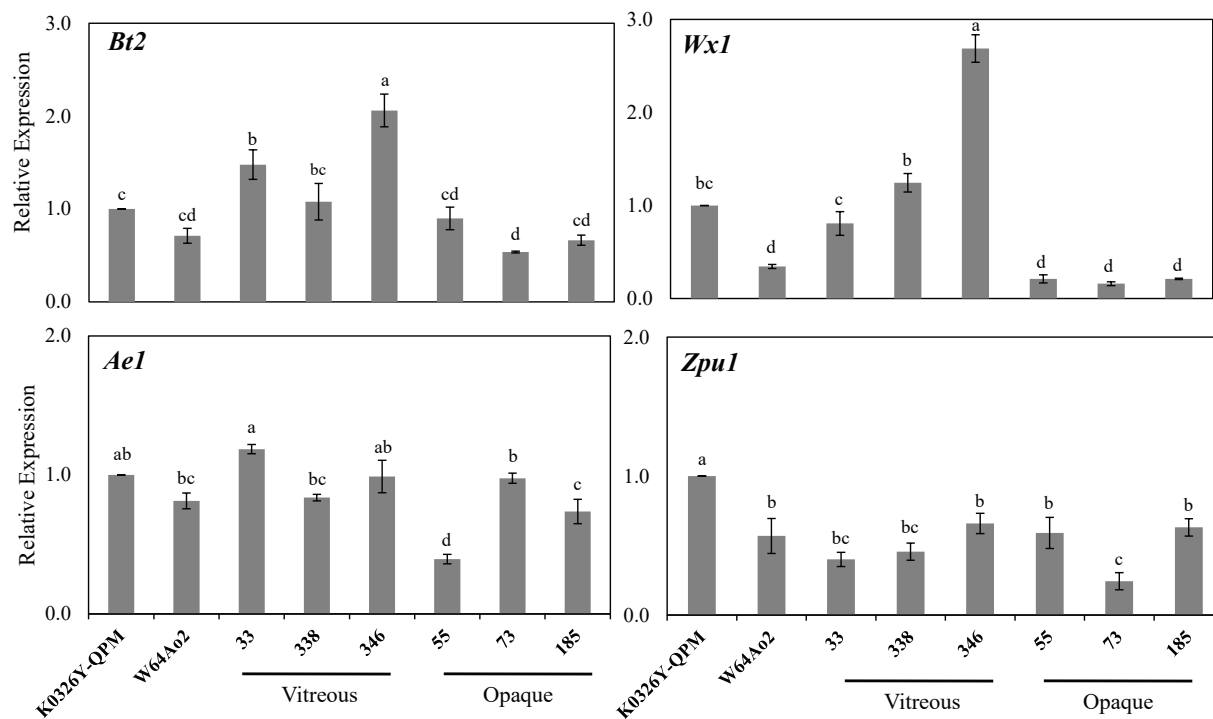
Figura 2. Expresión relativa de genes de síntesis de almidón en endospermo en desarrollo (30 DDP) de líneas de maíz contrastantes en vitrosidad y el genotipo de marcadores que flanquean QTLs para esta característica. El gen de la proteína relacionada a retinoblastoma 1 (*RRB1*) fue usado como referencia y los valores de expresión son relativos al de la línea K0326Y QPM. Letras diferentes indican diferencias significativas entre las líneas de maíz basado en la prueba de Fisher (P < 0.05).

Figure 2. Relative expression of starch biosynthesis genes in developing endosperm (30 DAP) of maize lines contrasting in vitreousness and the genotype of QTLs-flanking markers for this trait. The retinoblastoma-related protein-1 (*RRB1*) gene was used as a reference, and the expression values are relative to that of the K0326Y QPM line. Different letters indicate significant differences between the maize lines based on the Fisher test (P < 0.05).

(2021) also reported that the prolamin-like fraction that includes 27-kDa γ-zein showed higher accumulation in QPM lines compared to *opaque-2* genotypes after 30 and 45 DAP. Salazar-Salas *et al*. (2014) reported a significant correlation (r = 0.58, p < 0.01) between the levels of 27-kDa γ-zein and vitreousness in 283 RIL derived from the cross between K0326Y QPM and W64A*o2*. These results support the essential role of γ-zein in the vitreous phenotype of Quality Protein Maize (Wu *et al*., 2010), whose enhanced expression was associated with a duplication at the 27-kDa γ-zein locus (Liu *et al*., 2016).

The *Bt2* gene encodes for ADP-glucose pyrophosphorylase, a key enzyme in starch biosynthesis (Giroux and Hannah, 1994). The relative expression of this gene was not significantly different between the parental lines K0326Y QPM and W64A*o2* (Fig 2), which corresponded with the similar starch content observed in developing endosperm from these lines (Table 4). There were some differences in *Bt2* expression between the RILs, where the vitreous lines generally showed higher transcript levels than the opaque lines (Fig. 2). The over-expression of *Bt2* (~2.5-fold) was associated with a 7.5 % increase in starch content in developing (30 DAP) maize endosperm (Li *et al*., 2011). However, the higher transcript levels of *Bt2* observed in some vitreous lines were not associated with significant changes in starch content (Table 4). The *Wx1* gene maps close to the QTL for amylose and amylopectin on chromosome 9 (Bnlg127). *Wx1* encodes

for granule-bound starch synthase I (GBSSI), the enzyme responsible for amylose synthesis (Denyer *et al*., 2001). The *Wx1* expression was three-fold greater in K0326Y QPM than W64A*o2* and eight-fold higher in vitreous than opaque RILs (Fig. 2). These results are in agreement with the higher amylose content observed in K0326Y QPM and the vitreous RIL with respect to W64A*o2* and the opaque RILs (Table 4). The increased expression of *Wx1* may lead to a more significant accumulation and activity of GBBSI that results in the high amylose content associated with the vitreous phenotype.

The *Ae1* gene encodes for the starch branching enzyme IIb (SBEIIb), which catalyzes the formation of the α-1,6 glycosidic bonds during the synthesis of amylopectin (Nishi *et al*., 2001). Although the amylopectin content, in general, was higher in W64A*o2* and opaque RILs than K0326Y QPM and vitreous RILs (Table 4); there were no significant differences in *Ae1* expression between opaque and vitreous lines at 30 DAP (Fig. 2). However, the expression of this gene was higher in the opaque lines at an earlier stage of endosperm development (20 DAP) (data not shown) and may explain the higher amylopectin content of these lines. Jia *et al*. (2013) also found a higher expression of *Ae1* in W64A*o2* with respect to its normal counterpart W64A+ at 22 DAP, which could favor the production of starch granules with larger crystalline regions associated with the opaque phenotype.

The *Zpu1* gene encodes the pullulanase-type starch debranching enzyme, which hydrolyzes the α-1,6 glycosidic bonds of pullulan, a linear polymer of maltotriose units produced during starch metabolism (Nakamura *et al.*, 1996). The expression level of this gene in the endosperm of the parental line W64A*o2* was half of that in K0326Y QPM (Fig. 2), but there were no significant differences between the opaque and vitreous RILs.

Accumulation of starch granule-associated proteins

Starch granule-associated proteins (SGAP) from developing and mature endosperms (Fig. 3, Table 5) were analyzed because the GBSSI-encoding gene (*Wx1*) was found to be differentially expressed between the vitreous and opaque lines. The most abundant protein (~55 kDa) was GBSSI and showed more significant accumulation in developing endosperms from the vitreous lines (Fig. 3), which corresponds with the higher amylose content observed in
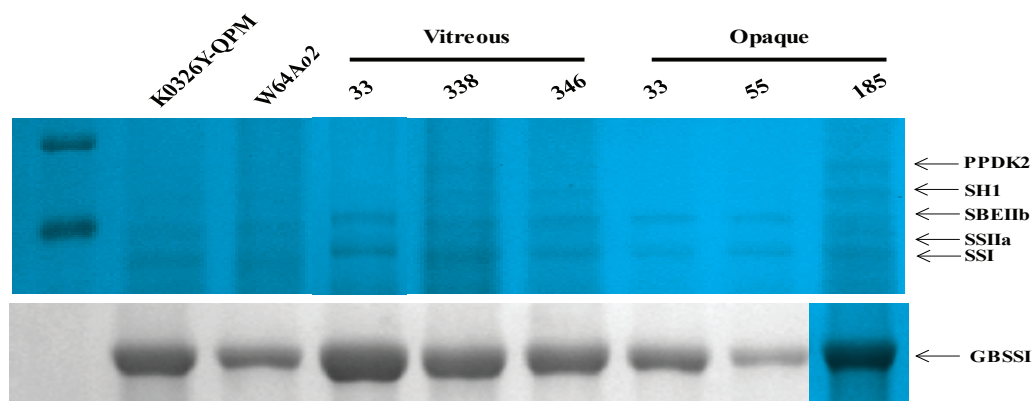

Figura 3. Separación por SDS-PAGE de proteínas asociadas al gránulo de almidón en endospermo en desarrollo (30 DDP) de líneas contrastantes en vitrosidad y el genotipo de marcadores que flanquean QTL asociados con esta característica. Los marcadores de peso molecular (kDa) se indican a la izquierda y la identidad de las proteínas se indica a la derecha. Las proteínas fueron identificadas por espectrometría de masas (Tabla 5). GBSSI, Almidón sintasa unida al gránulo I; SSI, Almidón sintasa I; SSIIa, Almidón sintasa IIa; SBEIIb, Enzima ramificadora de almidón IIb; SHI, Sacarosa sintasa I; PPDK2, Piruvato fosfato dicinasa 2.
Figure 3. SDS-PAGE analysis of starch granule-associated proteins from developing endosperm (30 DAP) of lines contrasting in vitreousness and the genotype of markers flanking QTLs for this trait. Molecular weight (kDa) standards are shown on the left, and the corresponding proteins are indicated on the right. Protein bands were identified by mass spectrometry (Table 5). GBSSI, Granule-bound starch synthase I; SSI, Starch synthase I; SSIIa, Starch synthase IIa; SBEIIb, Starch branching enzyme IIb; SH1 sucrose synthase 1; PPDK2, Pyruvate phosphate dikinase 2.

Tabla 5. Identificación por espectrometría de masas de proteínas asociadas al gránulo de almidón.
Table 5. Identification by tandem mass spectrometry of starch granule associated proteins.

Accession No.	Protein name	Theoretical Mw (kDa)	Observed Mw (kDa)	Sequence Coverage (%)	Peptides matched
A9Y0P1	Granule-bound starch synthase [*Zea mays*]	65.8	63.2	21.52	EALQAEVGLPVDR FSVDCNVVEPADVK FEPCGLIQLQGMR
O49064	Starch synthase I [*Zea mays*]	70.5	75.6	12.3	GAVVTADRIVTVSK EHKSSWEGLMK GIDLIQLIIPDLMR
Q6RG16	Starch synthase IIa, [*Zea mays*]	79.9	81.7	9.33	ITAGADVLVMPSR MLQHLEREHPNK APRDAALVRAEAEAGGK
O81387	Starch branching enzyme IIb [*Zea mays*]	90.6	89.6	17.9	VVLDSDAGLFGGFSR KAVMVPEGENDGLASR FIPGNNNSYDKCRR
K7V5Z8	Shrunken1 [*Zea mays*]	91.7	96.0	10.27	ELANLVIVAGDHGK LLPDAAGTTCGQR VIGTEHTDIIR
K7UZT6	Pyruvate, phosphate dikinase 2 [*Zea mays*]	96.0	105.6	21.63	EGEWLSLNGSTGEVILGK IAVDMVNEGLVER AWTAFPGSAQEGIGR

these samples (Table 4). The other SGAP corresponded to the enzymes starch synthase I (SSI), starch synthase IIa (SSIIa), starch branching IIb (SBEIIb), sucrose synthase 1 (SH1), and pyruvate phosphate dikinase 2 (PPDK2), which are involved in amylopectin synthesis and starch metabolism. Unfortunately, the low abundance of these proteins did not allow to register differences in accumulation between the vitreous and opaque lines. Other authors previously reported similar SGAP patterns in maize (Grimaud *et al*., 2008; Juárez-García *et al*., 2013; Salazar-Salas *et al*., 2014). PPDK and SH1 form complexes with some starch biosynthetic enzymes like SSIII, SSIIa, SBEIIa, and SBEIIb in maize amyloplasts and they may regulate the carbon partitioning between metabolic pathways (Hennen-Bierwagen *et al*., 2009).

CONCLUSIONS

The endosperm modification in QPM is associated with changes in the expression of starch biosynthesis genes, such as *Wx1* that showed higher transcript levels in the endosperm of the vitreous lines (K0326Y QPM, 33, 338, 346), which resulted in a more significant accumulation of GBSSI and consequently the synthesis of starch with a higher proportion of amylose during kernel development. As a result, starch granules with more amorphous regions may favor their packing and association, contributing to the vitreous phenotype. The information about the mechanisms involved in the modification of the *opaque-2* endosperm will facilitate the development of QPM genotypes with better agronomic and processing characteristics.

ACKNOWLEDGEMENTS

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