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Mechanisms associated with endosperm modification in quality protein maize

Mecanismos asociados con la modificación del endospermo en maíz de calidad proteínica

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ABSTRACT

Quality protein maize (QPM) combines the protein quality of the *opaque-2* (*o2*) mutant with a vitreous endosperm. These characteristics have allowed breeding programs worldwide to produce QPM genotypes that help alleviate malnutrition of people in developing countries from Africa, Asia and Latin America with a cereal-based diet. However, the development of these materials has been inefficient due to the limited knowledge about the molecular basis of the soft *o2* endosperm conversion into a vitreous phenotype in QPM. This conversion has been associated with an increase in small protein bodies rich in 27 kDa γ-zein, the synthesis of starch with a higher proportion of amylose and short-intermediate amylopectin chain branches that favors the compaction of the starch granules, as well as alterations in the amyloplast envelope that favors the interaction between starch granules and protein bodies. Additional studies about the mechanisms involved in the modification of the endosperm in QPM will contribute to produce materials with good agronomic characteristics and protein quality.

Keywords: *Zea mays* L.; endosperm modification; starch; zeins

RESUMEN

El maíz de calidad proteínica (MCP) combina la calidad proteínica de la mutante *opaco-2* (*o2*) con un endospermo vítreo. Estas características han permitido a los programas de mejoramiento alrededor del mundo producir genotipos MCP que ayudan a aliviar la malnutrición de la gente en países en desarrollo de África, Asia y América Latina con una dieta basada en cereales. Sin embargo, el desarrollo de estos materiales ha sido poco eficiente debido al limitado conocimiento acerca de las bases moleculares de la conversión del endospermo suave *o2* en un fenotipo vítreo en MCP. Esta conversión se ha asociado con el incremento en cuerpos proteínicos pequeños ricos en γ-zeína de 27 kDa, la síntesis de almidón con una mayor proporción de amilosa y ramificaciones de amilopectina cortas-intermedias que favorece la compactación de los gránulos de almidón, así como alteraciones en la envoltura de los amiloplastos que favorece la interacción entre gránulos de almidón y cuerpos proteínicos. Estudios adicionales sobre los mecanismos involucrados en la modificación del endospermo en MCP contribuirán a producir materiales con buenas características agronómicas y buena calidad proteínica.

Palabras clave: *Zea mays* L., modificación del endospermo, almidón, zeínas

INTRODUCTION

Maize (*Zea mays* L.) is the most important cereal with a global production of 1,162 million tons in 2020 (FAOSTAT, 2022). This cereal has a great social impact in developing countries where it is the main food staple. However, the most abundant proteins of maize (prolamins or zeins) are deficient in the essential amino acids lysine and tryptophan affecting the nutritional quality of the grain.

Mertz *et al.* (1964) found that the *opaque-2* (*o2*) mutation (Figure 1) almost doubles the lysine content in maize endosperm and improves the protein quality, but the use of this mutant in breeding programs was limited due to its poor agronomic performance associated with the opaque/soft endosperm and low seed density. Some years later, Paez *et al.* (1969) found that some segregating S_2 lines derived from opaque S_1 parents showed modified *o2* kernels (50% translucent or vitreous) whose lysine content was not different from the opaque kernels. Therefore, selection for hard endosperm was started. Researchers from the International Maize and Wheat Improvement Center (CIMMYT) in Mexico (Villegas *et al.*, 1992) and the University of Natal in South Africa (Gevers and Lake, 1992), developed a modified *o2* mutant or quality protein maize (QPM) (Figure 1) by recurrent backcrossing. This process required the simultaneous selection of kernels with normal texture and enhanced levels of essential amino acids. Thus, QPM combines the protein quality of *o2* with a vitreous endosperm and has better agronomic characteristics.

The QPM materials developed by the CIMMYT maize breeding program have been used worldwide as donors of *o2* modifiers to produce lines and hybrids adapted to each region. However, conventional QPM breeding involves the introgression of *o2* into a local adapted genotype that is sub-

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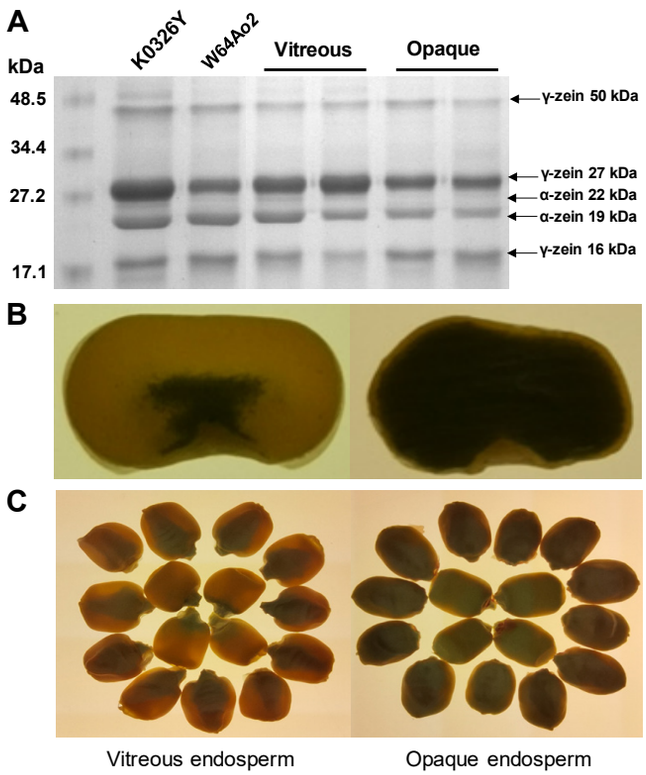

Figure 1. Zein profiles and endosperm phenotype of vitreous (QPM) and opaque maize genotypes. (A) SDS-PAGE separation of zein proteins. The molecular weight marker (kDa) is shown on the left and the different zein sub-fractions are indicate on the right. A greater abundance of 27-kDa γ-zein can be observed in the endosperm of vitreous lines compared to the opaque ones. Adapted from Vega-Alvarez *et al.* (2022). (B) Cross section of K0326Y (vitreous) and W64A*o2* (opaque) maize kernels viewed under white light. (C) Whole grains of K0326Y (vitreous) and W64A*o2* (opaque) viewed under white light.

Figura 1. Perfiles de zeínas y fenotipo del endospermo en genotipos de maíz vítreos (QPM) y opacos. (A) Separación por SDS-PAGE de proteínas zeínas. El marcador de peso molecular (kDa) se muestra a la izquierda y las diferentes subfracciones de zeínas se indican a la derecha. Se puede observar una mayor abundancia de γ-zeína 27 kDa en el endospermo de líneas vítreas comparado con las opacas. Adaptado de Vega-Alvarez et al. (2022). (B) Sección transversal de granos de maíz de las líneas K0326Y (vitrea) y W64Ao2 (opaca) vistos bajo luz blanca. (C) Granos enteros de maíz de las líneas K0326Y (vitrea) y W64Ao2 (opaca) vistos bajo luz blanca.

sequently used to pollinate a modifier donor, a process that requires several generations of backcrossing and self-crossing and the monitoring of high levels of lysine and tryptophan, the recessive *o2* mutant allele, and the modifiers. This lengthy and laborious strategy can take more than six years. The use of molecular markers has facilitated QPM breeding but this process could be more efficient if the mechanisms involved in the conversion of the soft *o2* endosperm into a vitreous phenotype were understood (Gibbon and Larkins, 2005). The main mechanisms proposed for this conversion include an increase in the accumulation of small protein bodies enriched in 27 kDa γ-zein (Wu *et al.*, 2010), the alteration of the structure and composition of starch due to the increase in the proportion of amylose and short-intermediate amylopectin chain branches (Gibbon *et al.*, 2003; Salazar-Salas *et al.*, 2014) and the loss of the amyloplast envelope due to a reduction in non-polar carotenoids (Wang *et al.*, 2020). The aim of the present review is to provide the current advances about the molecular mechanisms associated with the modification of the vitreous endosperm in QPM.

MAIZE KERNEL COMPOSITION

The major components of the maize kernel are starch (64 - 78 %) and proteins (8 - 15 %). Starch is mainly found in the endosperm while proteins are more abundant in the germ. The remaining components of the grain consist of lipids (4.0 - 4.9 %), ashes (1 - 3 %) and fiber (1 - 2 %) (Serna-Saldivar, 2019). Starch and proteins influence the physicochemical and structural characteristics of the kernel, highlighting the importance of these components.

Starch biosynthesis

Starch is the main carbon reserve in cereals (70 - 80 %) and is mainly responsible for their energetic and economic value. It is formed by two homopolysaccharides: amylose, an essentially linear molecule formed by glucose units linked by α-(1,4) glycosidic bonds, and amylopectin, a molecule formed by linear portions of glucose linked by α-(1,4) glycosidic bonds and ramifications linked by α-(1,6) bonds (Pfister and Zeeman, 2016).

In cereals, starch is mainly found in endosperm cells forming granules and its biosynthesis is carried out by the coordinated action of multiple enzymes: ADP-Glucose pyrophosphorylase (AGPase), starch synthases (GBSS, SS), starch branching enzymes (SBE) and starch debranching enzymes (DBE) (Figure 2) (Comparot-Moss and Denyer, 2009). This process begins with the enzyme AGPase that produces ADP-glucose in the cytosol, which is transported into the plastid and serves as a substrate for starch synthase enzymes (Pfister and Zeeman, 2016). The granule bound starch synthase I (GBSSI) is encoded by the *waxy* locus in cereals and is responsible for the elongation of amylose, being essential within the granule matrix. The soluble starch synthases (SSI, SSII, SSIII and SSIV) are exclusively involved in the synthesis of amylopectin chains and are associated with the starch granules. Genetic and biochemical studies indicate that each SS isoform has different properties and a different role in amylopectin synthesis. The starch branching enzyme (SBE) generates α-(1,6) glycosidic bonds after breaking the α-(1,4) bond and transferring the chain to the C6 from a glucose residue of another chain, forming the branched structure of the amylopectin molecule (Huang *et al.*, 2021). Two isoforms of branching enzymes are expressed in the endosperm of cereals, branching enzyme I (SBEI) and branching enzyme II (SBEII), which differ in the length of the transferred glucan chain and substrate specificity; SBEI shows greater affinity for amylose and transfers longer chains than SBEII, which transfers shorter chains and has greater affinity for amylopectin (Sawada *et al.*, 2018).

The starch debranching enzyme (DBE) catalyzes the hydrolysis of glycosidic bonds α-(1,6). In higher plants the-

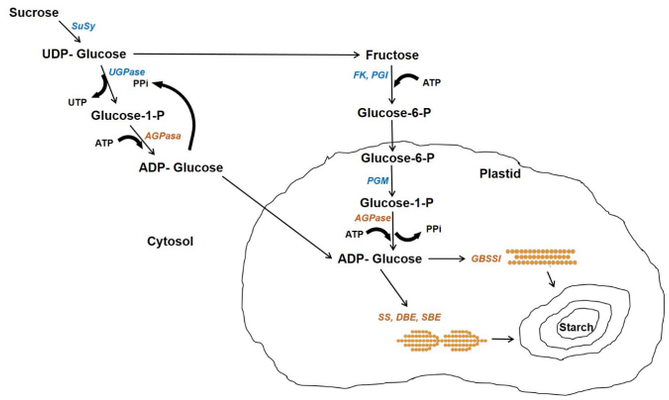

Figure 2. Starch biosynthesis pathway in the endosperm of cereals. The cytosol and plastid are indicated. Enzymes are indicated in italics: Susy, sucrose synthase; UGPase, UDP glucose pyrophosphorylase; PGM, phosphoglucomutase; FK, fructokinase; PGI, phosphoglucose isomerase; PPiase, pyrophosphatase; AGPase, ADP-glucose pyrophosphorylase; GBSSI, granule bound starch synthase; SS, starch synthase; SBE, starch branching enzyme. Adapted from Comparot-Moss and Denyer (2009).

Figura 2. Ruta de síntesis de almidón en el endospermo de cereales. Se indican el citosol y el plástido. Las enzimas están indicadas en itálicas: Susy, sacarosa sintasa; UGPasa, UDP glucosa pirofosforilasa; PGM, fosfoglucomutasa; AGPasa, ADP-glucosa pirofosforilasa; GBSSI, almidón sintasa unida al gránulo; SS, almidón sintasa; SBE, enzyma ramificadora de almidón. Adaptada de Comparot-Moss and Denyer (2009).

re are two types of DBE and they are defined according to the specificity of their substrate: debranching enzymes of the isoamylase type and the pullulanase type. Isoamylase breaks down amylopectin and phytoglycogen branches, while pullulanase acts on pullulans and amylopectin but not phytoglycogen (Robyt, 2009).

Synthesis of zeins

The most abundant storage proteins in maize kernels are prolamins (soluble in alcohol) or zeins (50-55%), followed by glutelins (soluble in alkaline solutions) (35-40%), while albumins (soluble in water) and globulins (soluble in saline solutions) account for less than 10% of the total proteins (Larkins, 2019; Sethi *et al.*, 2021). Because zeins are the major proteins in the kernel, total proteins are typically divided into zeins and non-zeins. Zeins are synthesized by membrane-delimited polyribosomes and transported within the lumen of the rough endoplasmic reticulum, where they assemble into protein bodies (Lending and Larkins, 1989). Protein bodies are small spherical structures made up of a protein matrix surrounded by a simple membrane; they contain at least four types of zeins (α-, β-, γ-, δ-), which are classified based on their solubility and structural similarities as α-zeins (22 and 19 kDa), β-zeins (14 and 16 kDa), γ-zeins (16, 27 and 50 kDa) and δ-zeins (10 kDa) **(**Figure 1A) (Coleman and Larkins, 1999). Immunolocalization studies showed that γ- and β-zeins are generally located in the peripheral region of protein bodies, while α-zeins are located in the internal region of these structures (Lending and Larkins, 1989).

The *o2* mutant has a defective transcription factor that regulates the expression of α-zeins and depending on the genetic background the content of these proteins can be reduced more than 50% (Figure 1A) (Kodrzycki *et al.*, 1989). Zeins are deficient in some essential amino acids (lysine and tryptophan) and the reduced synthesis of these proteins in *o2* results in higher levels of more nutritionally balanced non-zeins (Lopez-Valenzuela *et al.*, 2004) and free amino acids in the endosperm (Pineda-Hidalgo *et al.*, 2011). The use of RNA interference (RNAi) to block the expression of α-zeins has also shown to increase the levels of lysine and tryptophan in maize endosperm (Huang *et al.*, 2006), which avoids the negative characteristics and limitations of the recessive *o2* mutant. The Cas9/CRISPR technology represents another biotechnological alternative to reduce or remove zein gene expression as a strategy to increase the levels of proteins with a better balance of essential amino acids (Jiang *et al.*, 2022).

QUALITY PROTEIN MAIZE AND ITS IMPORTANCE IN HUMAN NUTRITION

Malnutrition is a problem that affects more than 828 million people worldwide, 98% of which are from developing countries, mainly Africa, Asia, Latin America and the Caribbean, and includes 150 million children (FAOSTAT, 2022). People from developing countries with a cereal-based diet have a high risk of protein and lysine deficiency (Muleya *et al.*, 2022), although this deficiency can occur in any people with a diet based on cereals. Several investigations have documented the benefits of QPM in human nutrition, highlighting its potential to mitigate problems associated with protein-energy deficiency in children under 5 years of age, the elderly and pregnant women, considered the most vulnerable groups (Hossain *et al.*, 2019).

The consumption of QPM instead of normal maize, increased 12 - 15% the weight growth rate in infants and young children, with mild to moderate undernutrition (Akalu *et al.*, 2010; Gunaratna *et al.*, 2010). QPM has also a higher content of phenylalanine and isoleucine, suggesting it can be included in the family diet to reduce the risk of inadequate protein intake (Gunaratna *et al.*, 2019). Tortillas from nixtamalized and extruded QPM flours showed higher nutritional indicators (C-PER, protein digestibility, PER, NPR, PDCAAS) than those from normal maize, suggesting they may have a positive effect on the nutritional status of people from countries where these products are widely consumed (Gutiérrez-Dorado *et al.*, 2008). Desalegn *et al.* (2015) showed that QPM based complementary foods have good sensory acceptability and can help meet the minimum recommended daily dose of energy (370 kcal) and protein (10.9 g) for children aged 6 - 36 months, as well as two thirds of the recommended iron and zinc daily dose and up to 50 % of vitamin A. The supplementation of malnourished young children (4 - 6 years old) with QPM-based biscuits reduced the percentage of anemic subjects from 63.3 % to 16.6 % and the prevalence of severe underweight from 23.3 % to 0 % (Grover *et al.*, 2020).

In recognition of the great potential of QPM to improve human nutrition in poor countries where maize is a staple food, Dr. Surinder K. Vasal and Dr. Evangelina Villegas from

CIMMYT were awarded with the World Food Prize in 2000 (Cordova, 2001).

ADVANCES IN THE DEVELOPMENT OF QPM

Despite the QPM nutritional value and agronomic performance, the cultivation and adoption of these materials on a large scale has not been achieved, mainly due to the low availability of genetically diverse and competitive QPM hybrids compared to non-QPM / normal hybrids, the lack of information about their health benefits and government incentives (Hossain *et al.*, 2018; Maqbool *et al.*, 2021). Nevertheless, breeding programs have been implemented around the world with the purpose of producing new and better QPM genotypes; they have mainly used QPM donors from CIMMYT in Mexico (Cordova, 2001; Vivek *et al.*, 2008). Until 2015, more than 167 QPM varieties were released worldwide, 53 % in Africa, 25 % in Latin America and 22 % in Asia (Twumasi-Afriyie *et al.*, 2016). Some of these QPM genotypes are listed in Table 1. Conventional breeding strategies such as recurrent selection were initially used for QPM development, but in the last decades a widely used strategy to develop these materials is molecular marker-assisted breeding and most of the studies have used SSR markers (*e.g.* phi 057, phi 112 and umc 1066) located within the *o2* gene on the short arm of chromosome 7 (www.maizegdb.org) (Maqbool *et al.*, 2021). Some examples include the QPM version of the line V25 derived from the cross V25 × CML176 (QPM), which shows an increase in tryptophan content (0.85 %) and maintains a hard endosperm (Babu *et al.*, 2005), as well as Vivek QPM-9 (VQL 1 × VQL 2) that contains 41 % more tryptophan and 30 % more lysine than the original hybrid (Vivek Hybrid-9) (Table 1) (Gupta *et al.*, 2013).

Other researchers have focused on developing new QPM genotypes with certain agronomic and gastronomic characteristics, but always seeking to maintain the nutritional quality of *o2*. For instance, the Zhao OP-6 /*o2o2* corn was generated by introducing the *o2* allele into the Zhao OP-6 waxy corn to produce a waxy QPM line intended for the Chinese market, where waxy corn is widely used in food processing because of its high viscosity and digestibility (Zhou *et al.*, 2016). Quality Protein Popcorn (QPP) was developed recently, which showed a higher lysine content compared to its parent elite line and maintained its bursting capacity, demonstrating the potential use of QPM for the production of grains with specific functional characteristics and good protein quality (Ren *et al.*, 2018). Since most of the efforts to develop QPM have been based on the use of molecular markers that co-inherit with the o2 phenotype, improving the understanding of the molecular basis of endosperm modification could help to develop these materials more efficiently.

MECHANISMS ASSOCIATED WITH ENDOSPERM MODIFICATION IN QPM

Increased accumulation of γ-zein proteins

One of the first biochemical changes observed in QPM genotypes was an increase in γ-zein content (2 to 5 times) compared to *o2* (Figure 1A) and normal maize (Wallace *et al.*, 1990). Immunolocalization studies suggested that γ-zein initiates the formation of protein bodies (Lending and Larkins, 1989), which is supported by the fact that inhibition of the 27 kDa γ-zein encoding gene reduces significantly the number of protein bodies, while the inhibition of 19 and 22 kDa α-zeins decreases the diameter of these structures (Guo *et al.*, 2013). The γ-zeins are highly linked with disulfide bonds and it has been hypothesized that the covalent bonds of γ-zeins and other cysteine rich proteins promote the formation of a protein network around starch granules (Lopes and Larkins, 1991). Therefore, it has been proposed that an increase in the number of protein bodies and their compaction among the starch granules is, at least partially, responsible for the modification of the endosperm (Figure 3A). A genetic analysis using recombinant inbred lines (RIL) derived from the cross of Pool 33 (QPM) and W64A*o2* (soft endosperm), identified two *loci* associated with endosperm modification; these *loci* were located on the long arm of chromosome 7, one near the centromere linked to a 27 kDa γ-zein *locus* and the other near the telomere (Lopes *et al.*, 1995). Holding *et al.* (2008) identified 7 *loci* associated with *o2* modifier genes (*mo2*) in an F2 progeny from the cross of K0326Y-QPM and W64A*o2*; two major *loci* located on chromosomes 7 and 9 explained 40 % of the phenotypic variation and were linked to 27 kDa γ-zein and starch synthesis genes, respectively. Holding *et al.* (2011) developed RIL from the F2 progeny and the kernels were characterized for vitreousness, density and hardness; genetic mapping with these RIL identified *loci* on chromosomes 1, 7 and 9, confirming their linkage with γ-zein and starch biosynthesis genes.

Several studies have focused on the 27 kDa γ-zein locus to understand the mechanism of *o2* modifier genes. There is more than one gene for 27 kDa γ-zein in this *locus*, which are assigned depending on the genotype and are called A and B, and together form the AB *locus* (Das *et al.*, 1990). The AB locus of γ-zeins is not stable and can be rearranged to form A and B, called rA and rB, respectively (Das *et al.*, 1990; Das *et al.*, 1991). Because all known QPM lines contain the A and B genes, and all F2 progeny from a cross of a QPM and an *o2* mutant contain the AB locus, it has been hypothesized that the AB locus is necessary for endosperm modification, although it is also emphasized that this locus by itself is not sufficient to achieve such modification (Lopes *et al.*, 1995).

Although the molecular mechanism of the *mo2* genes is not fully understood, evidence suggests that a greater stability of the γ-zein mRNA and/or a higher transcription rate may be responsible for a larger accumulation of the 27 kDa γ-zein protein in modified *o2* genotypes. Geetha *et al.* (1991) suggested that the role of *mo2* genes is to increase the stability of γ-zein mRNA and protein synthesis. Or *et al.* (1993) suggested that the stability of the A gene mRNA initiates enhanced 27 kDa γ-zein protein synthesis. Burnett and Larkins (1999) found that the A:B ratio of γ-zein mRNA in *mo2* endosperms was more than 40:1, compared to a 1:1 ratio for normal maize and 3:1 for *o2*, indicating that these relationships can result from different transcription rates of the genes A and B.

Table 1. *Development of QPM genotypes.*
Tabla 2. *Desarrollo de genotipos QPM.*

Name	Pedigree/Background	Gene(s) introgressed	Country	Reference
NB-Nutrinta (OPV)	Poza Rica 8763	*o2*	Nicaragua	Cordova (2001)
HQ INTA-993 (hybrid)	(CML144 × CML159) CML176	*o2*	Nicaragua	Cordova (2001)
HB-PROTICTA (hybrid)	(CML144 × CML159) CML176	*o2*	Guatemala	
HQ-61 (hybrid)	(CML144 × CML159) CML176	*o2*	El Salvador	
HQ-31 (hybrid)	(CML144 × CML159) CML176	*o2*	Honduras	
ICA- (hybrid)	(CML144 × CML159) CML176	*o2*	Colombia	
FONAIAP (hybrid)	(CML144 × CML159) CML176	*o2*	Venezuela	
BR-473, BR-451 (OPV)		*o2*	Brazil	Cordova (2001)
INIA- (hybrid)	CML161 × CML165	*o2*	Peru	Cordova (2001)
HQ-2000 (hybrid)	CML161 × CML165	*o2*	Vietnam	Cordova (2001)
Zhongdan 9409 (hybrid)	Pool 33 × Temp QPM	*o2*	China	Cordova (2001)
QUIAN2609 (hybrid)	Tai 19/02 × CML171	*o2*	China	Cordova (2001)
Susuma (OPV)	Across 8363SR		Mozambique, Senegal	Krivanek *et al.* (2007)
Longe-5 'Nalongo' (OPV)	Across 8363SR		Uganda	Krivanek *et al.* (2007)
Obatanpa (OPV)	Across 8363SR		Benin, Burkina Faso, Cameroon, Cote d'Ivoire, Ghana, Guinea, Malawi, Mali, Nigeria, Senegal, South Africa, Togo	Krivanek *et al.* (2007)
Lishe-K1(OPV)	Across 8363SR		Tanzania	Krivanek et al. (2007)
EV 99 QPM (OPV)			Cote d'Ivoire, Nigeria, Senegal, Togo	Krivanek *et al.* (2007)
KH500Q (hybrid)	(CML144 × CML159) CML181	*o2*	Kenya	Krivanek *et al.* (2007)
BHQP542 (hybrid)	(CML144 × CML159) CML176	*o2*	Ethiopia	Krivanek *et al.* (2007)
MHQ138 (hybrid)	(CML144 × CML159) Pool15Q	*o2*	Ethiopia	Jilo (2022)
BHQPY545 (hybrid)	CML181 × CML165	*o2*	Ethiopia	Jilo (2022)
QS-7705 (hybrid)		*o2*	South Africa	Krivanek *et al.* (2007)
GH-132-28 (hybrid)	P62, P63	*o2*	Ghana	Krivanek *et al.* (2007)
ZS261Q (hybrid)	(CZL01006 × CML176) × (CZL01005 × CML181)	*o2*	Zimbabwe	Krivanek *et al.* (2007)
441C (hybrid)	CML142 × CML116	*o2*	Mexico	Cordova (2001)
H-551C (hybrid)	CML142 × CML150	*o2*	Mexico	Cordova (2001)
H-553C (hybrid)	(CML142 × CML150) CML176	*o2*	Mexico	Cordova (2001)
H-519C (hybrid)	(CML144 × CML159) CML170	*o2*	Mexico	Cordova (2001)
H-368EC (hybrid)	CML186 × CML149	*o2*	Mexico	Cordova (2001)
H-369EC (hybrid)	CML176 × CML186	*o2*	Mexico	Cordova (2001)
V-537C (OPV)	Poza Rica 8763	*o2*	Mexico	Gómez-M *et al.* (2003)
V-538C (OPV)	Across 8762			
H-374C (hybrid)	(CML176 × CML142) CML186	*o2*	Mexico	Noriega González *et al.* (2011)
H-564C (hybrid)	(LT158 × LT159) LT160	*o2*	Mexico	Sierra Macías *et al.* (2011)
V556AC (OPV)		*o2*	Mexico	Twumasi-Afriyie *et al.* (2016)
ZAPATA 3		*o2*	Mexico	Twumasi-Afriyie *et al.* (2016)
ZAPATA 9		*o2*	Mexico	Twumasi-Afriyie *et al.* (2016)
V25 QPM (line)	V25 × CML176	*o2*	India	Babu *et al.* (2005)
Vivek QPM-9 (hybrid)	VQL1 (CM212 × CML180) × VQL2 (CM145 × CML170)	*o2*	India	Gupta *et al.* (2009); Gupta *et al.* (2013)

Name	Pedigree/Background	Gene(s) introgressed	Country	Reference
BC2F4-1 (line)	QCL3024 (*o16*) × QCL5019 (*wx*) and QCL5008 (*wx*)	*o16*	China	Yang *et al.* (2013)
BQPM9 (line) BQPM10 (line) BQPM11 (line) BQPM12, BQPM16 (line) BQPM13, BQPM14 (line) BQPM15 (line) BQPM17 (line)	(B99 × CLQ 06901) B99 (B99 × CLRQ 00502) B99 (B100 × CLQ 06901) B100 (CLQ 06901 × B98) B98 (CLQ 06901 × B97) B97 (B91 × CLQ 06901) B91 (CLQ 06901 × B113) B113	*o2*	USA	Worral *et al.* (2015)
ZPL 3 QPM (line) ZPL 5 QPM (line)	ZPL 3 × CML144 ZPL5 × CML144	*o2*	Serbia	Kostadinovic *et al.* (2016)
Zhao OP-6/*o2o2* (line)	Zhao OP-6 × QPM CA339 (pool33)	*o2*	China	Zhou *et al.* (2016)
BML-7 QPM (line)	BML-7 × CML-186	*o2*	India	Krishna *et al.* (2017)
CBML6 QPM (line) CBML7 QPM (line) DHM117 (hybrid)	BML6 × CML181 BML7 × CML181 CBML6 × CBML7	*o2*	India	Surender *et al.* (2017)
HM4 QPM (line) HM8 QPM (line) HM9 QPM (line)	HKI323 × HKI161 HKI1105 × CMLI61 HKI1128 × HKI193-1	*o2*	India	Hossain *et al.* (2018)
V238AC (OPV)	Comiteco race (yellow) × CML-172	*o2*	Mexico	Coutiño Estrada and Vázquez Carrillo (2018)
Quality Protein Popcorn (QPP) (lines)	CML154Q × (P2, P3, P9) Tx807 × P2 K0326Y × (P3, P7)	*o2*	USA	Ren *et al.* (2018)
QCL8006-1 (line) QCL8006-2 (line)	QCL3024 (*o16*) × Taixi19 (*o2*) and QCL5019 (*wx*)	*o2/o16*	China	Wang *et al.* (2019)
HM5-A (hybrid) HM12-B (hybrid)	(HKI1344 × PMI-102-*o2o16*) × (HKI1348-6-2 × PMI-102-*o2o16*) (HKI1344 × PMI-102-*o2o16*) × HKI1378 × PMI-102-*o2o16*)	*o2/o16*	India	Chand *et al.* (2022)
V56AC (OPV)	Oloton race (yellow) × CML-172	*o2*	Mexico	Coutiño Estrada *et al.* (2022)

OPV: Open pollinated variety.

These results are consistent with a model in which the two *loci* associated with *mo2* genes influence the expression of γ-zein genes through different mechanisms: one affects the transcription of the γ-zein *locus* and the other influences the stability of the γ-zein RNA. Holding *et al.* (2011) evaluated the expression in developing endosperm (18 days after pollination, DAP) of QPM lines contrasting in vitreousness, reporting a higher expression of the 27 kDa γ-zein gene and greater accumulation of the protein in vitreous QPM lines compared to *o2* lines (Figure 1A). Wu *et al.* (2010) used RNAi to block the expression of 27 kDa γ-zein in the CM105*mo2* maize line; and found that RNAi caused the reversal of the vitreous phenotype to *o2*, demonstrating that 27 kDa γ-zein plays an essential role in the modification of the endosperm in QPM. Similar findings were reported by Yuan *et al.* (2014) who used γ-radiation mutagenesis to identify genes related to the modification of the *o2* mutant; they observed a generalized decrease in α-zeins and an increase in γ-zeins (27 and 50 kDa) in the K0326Y-QPM line compared to W64A+. The authors proposed that the 27 kDa γ-zein plays an important role in the formation of protein bodies and that the 50 kDa γ-zein, despite being in a smaller proportion, could also be involved in endosperm modification.

A genetic analysis in QPM RILs identified a *locus* (qγ27) on chromosome 7 that results from the duplication of the 27 kDa γ-zein gene and causes an increase in gene expression and the synthesis of 27 kDa γ-zein in QPM and wild-type lines, confirming that the improved expression of 27 kDa γ-zein is critical for endosperm modification in QPM (Liu *et al.*, 2016). The higher expression of 27 kDa γ-zein causes that QPM endosperm accumulates a greater amount of small protein bodies, which are suggested to allow the formation of a more rigid vitreous matrix that resembles a wild type of maize endosperm **(**Figure 3A) (Wu *et al.*, 2010).

Alteration in starch composition and structure

The non-zein fraction in maize endosperm includes metabolic enzymes that may also play a role in QPM endosperm modification. Gibbon *et al.* (2003) performed a gel-based proteomic analysis of non-zeins in maize near isogenic lines contrasting in vitreousness (CM105+, CM105*o2* and CM105*mo2*), and found that the vitreous QPM line showed an increased accumulation of the enzyme granule-bound starch synthase I (GBSSI), which is responsible for the synthesis of amylose. The authors also found that amylopectin in the vitreous endosperms showed a higher proportion of short branches

compared to that of normal and *o2*. These alterations in starch structure may increase the proportion of amorphous regions at the surface of starch granules, which favors their compaction and the vitreous phenotype (Figure 3A). These results suggested that starch biosynthetic enzymes may play an important role in endosperm modification.

Genetic analyses of the cross between K0326Y-QPM and W64A*o2* found a locus for vitreousness on chromosome 9 near genes involved in starch biosynthesis (Holding *et al.*, 2008; Holding *et al.*, 2011). The biochemical characterization of this locus, using RILs derived from the same cross, showed that starch from vitreous mature endosperms had higher levels of amylose and lower crystallinity compared to starch from opaque endosperms, which was associated with lower gelatinization enthalpy (Salazar-Salas *et al.*, 2014). This behavior was also observed by Juárez-García *et al.* (2013) who reported lower enthalpy values in starches from vitreous lines compared to those from opaque lines at the mature state. These results could be explained by the higher proportion of amylose and short branches of amylopectin in starch from the vitreous lines. Short branches of amylopectin can reduce crystal formation, while a higher proportion of long chains can form more organized crystals that require higher temperature and gelatinization enthalpy (Jane *et al.*, 1999). These studies suggest that alterations in the amylopectin structure play an important role in the modification of the endosperm.

Wu *et al.* (2015) reported that SSSIII may affect pullulanase activity and indirectly influence the vitreousness of the kernel by altering the distribution and length of the amylopectin glucan chains. Soluble starch synthase I (SSSI) produces short chains with degrees of polymerization (GP) of 8 - 12, while SSSII and SSSIII isoforms seem to be involved in the formation of intermediate (GP 13 - 25) and long (GP > 30) chains, respectively (Nakamura *et al.*, 2005). A higher proportion of amylopectin intermediate chains (GP 10 - 24) and a decrease in the proportion of chains with GP of 25 - 40 was observed in starch from K0326Y-QPM and vitreous RILs with respect to starch from W64A*o2* and opaque RILs, which was associated with a higher proportion of amorphous regions in the starch granules that favors their compaction adopting polygonal shapes (Gibbon *et al.*, 2003; Salazar-Salas *et al.*, 2014). This provides a mechanism that complements the one associated with an increase in small protein bodies rich in γ-zein (27 kDa) that fill the spaces between the starch granules creating the vitreous phenotype **(**Figure 3A).

Genetic mapping of starch physicochemical properties in RIL derived from K0326Y QPM and W64A*o2* identified three *loci* on bins 4.05, 5.04, and 9.03 close to the starch biosynthesis genes *Brittle-2* (*Bt2*), *Amylose extender-1* (*Ae1*), and *Waxy-1* (*Wx1*), respectively (Vega-Alvarez *et al.*, 2022); the analysis of gene expression in developing endosperm (30 days after pollination, DAP) showed that the transcript levels of *Wx1* were significantly higher in K0326Y QPM and vitreous RILs compared with W64A*o2* and opaque lines, which corresponded to a greater GBSSI and amylose accumulation in the vitreous lines at the same developmental stage. These results are in agreement with those reported in mature endosperm (Salazar-Salas *et al.*, 2014) and confirms an important role for GBSSI in the modification of the QPM endosperm. Jia *et al.* (2013) analyzed the expression in developing endosperm (22 DAP) of W64A*o2* and its normal counterpart and found a lower expression of *Wx1* in the opaque mutant. This study also revealed that the expression of genes encoding pullulanase (*Zpu1*) and starch branching IIb (SBEIIb) enzymes was higher in W64A*o2* than W64A+. The regulation of these genes may change the proportions of amylose and the branching patterns of amylopectin in the starch granules of the *o2* mutant contributing to the soft endosperm. Gonzalez-Nuñez (2022) analyzed the activity of GBSSI and SBEIIb in developing endosperms (28 DAP) of K0326Y-QPM, W64A*o2* and RIL contrasting in vitreousness; the GBSSI activity was higher in the endosperm of the vitreous lines and was associated with a higher proportion of amylose, whereas the activity of SBEIIb was higher in opaque lines that showed higher levels of amylopectin. These results support the hypothesis that endosperm modification in QPM is associated with the synthesis of starch with a higher proportion of amylose, which may facilitate the packing of the starch granules resulting in the vitreous phenotype (Figure 3A).

Modulation of carotenoid composition in amyloplast envelope

Wang *et al.* (2020) identified *Ven1* as a major QTL influencing the vitreous phenotype in the mature maize kernel. *Ven1* encodes for the enzyme β-carotene hydroxylase 3, which modulates the composition of carotenoids in the amyloplast envelope. They observed that in the opaque endosperm is a dysfunctional *Ven1* allele that decreases the content of polar carotenoids and increases that of non-polar carotenoids in the amyloplast envelope, which provides greater stability to this structure that under normal circumstances disappears during kernel desiccation. The non-disruption of the amyloplast envelope results in a poor interaction between the protein bodies and the starch granules, leading to a soft endosperm (Figure 3A).

Amelioration of the stress response by increasing the energy availability

The development of the *o2* endosperm involves a stress response that reduces the energy levels and affects ATP-dependent processes such as zein proteins synthesis (Li *et al.*, 2020). This may be due to a reduction in the expression of pyruvate phosphate dikinase (PPDK1), affecting glycolysis and the energy production. Holding *et al.* (2008) and Holding *et al.* (2011) identified several differentially upregulated genes in QPM, including pyrophosphate-dependent fructose-6-phosphate 1-phosphotransferase (PFPα), a non-ATP-dependent glycolytic enzyme. It was proposed that the higher activity of PFPα in QPM compensates the reduced availability of ATP in *o2* endosperm (Guo *et al.*, 2012). A cytosolic PPDK2 was also identified by these authors as an ATP-independent glycolytic enzyme. Li *et al.* (2020) also found PFPα as a candi-

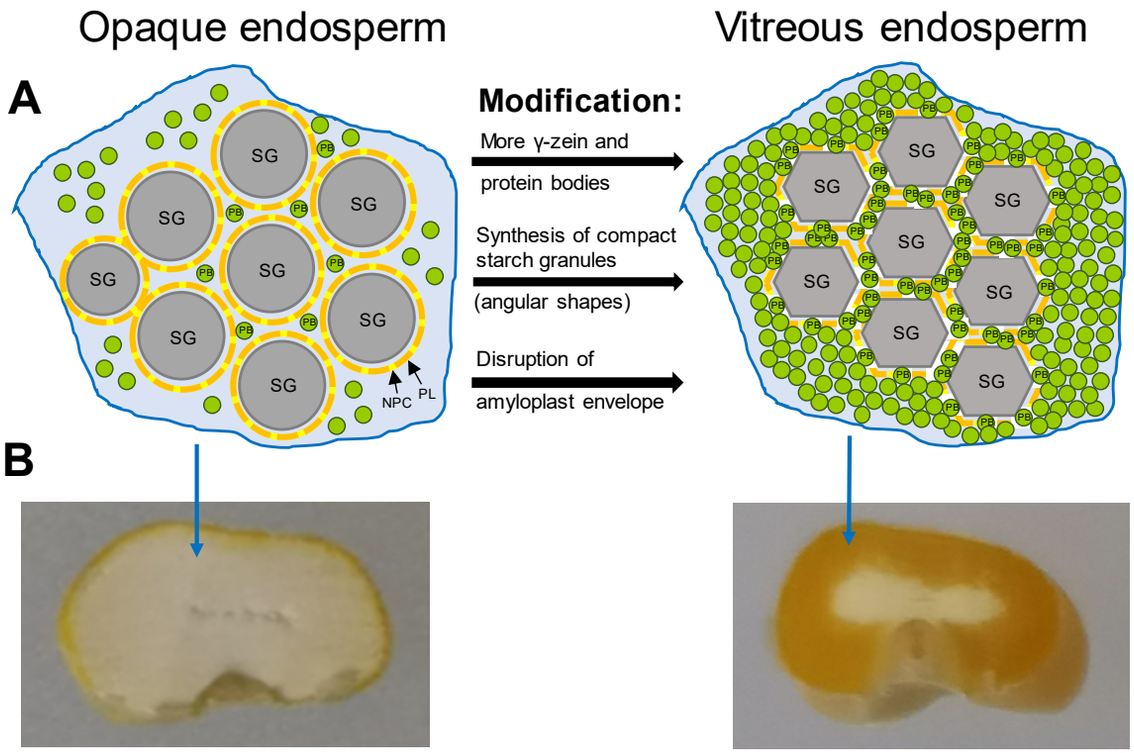

Figure 3. Vitreous endosperm formation in quality protein maize. (A) Representation of the main mechanisms associated with endosperm modification: Mechanism associated with an increase in protein bodies (PB) rich in 27 kDa γ-zein that fill the spaces between starch granules (SG); Mechanism associated with changes in the composition and morphology of the starch granules that favors their compaction and interaction with PBs; Mechanism associated with the disruption of the amyloplast membrane that favors the interactions between starch granules and PBs. PL, Phospholipids; NPC, Non-polar carotenoids. Adapted from Wang *et al.* (2020). (B) Cross section of mature kernels from *opaque-2* and QPM genotypes showing the extent of vitreous endosperm.
Figura 3. Formación del endospermo vítreo en el maíz de calidad de proteínica. (A) Representación de los principales mecanismos asociados con la modificación del endospermo: Mecanismo asociado con un incremento en cuerpos proteínicos (PB) ricos en γ-zeína 27 kDa que llenan los espacios entre los gránulos de almidón (SG); Mecanismos asociado con cambios en la composición y morfología de los gránulos de almidón que favorece su compactación e interacción con PBs; Mecanismo asociado con la disrupción de la membrana del amiloplasto que favorece las interacciones entre los gránulos de almidón y PBs. PL, fosfolípidos; NPC, carotenoides no polares. Adaptada de Wang et al. (2020). (B) Sección transversal de granos maduros de genotipos opaco-2 y MCP mostrando la extensión del endospermo vítreo.

date gene for endosperm modification in QPM and identified cytosolic enolase (ENO) as another ATP-independent glycolytic enzyme. Thus, the increased synthesis of enzymes that do not require ATP for glycolysis in QPM provides energy by a mechanism that is repressed in *o2* endosperm.

CONCLUSIONS

There have been important advances in the understanding of the mechanisms associated with endosperm modification in QPM. However, the application of this information for the efficient development of QPM materials is difficult due to the multiple mechanisms involved in the creation of the vitreous endosperm. So far, the increased accumulation of 27 kDa γ-zeins seems to have the major contribution to the vitreous phenotype, which is complemented with the alterations in the composition and structure of the starch granules that favor their compaction, as well as with alterations in the composition of the amyloplast envelope that result in the degradation of this structure during endosperm desiccation, allowing a better interaction between protein bodies and starch granules. These processes may not be possible without the availability of energy provided by enzymes that enhance the non-ATP-dependent glycolytic flux.

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CONFLICTS OF INTEREST

The authors have no conflict of interest to declare.

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