



Review article

## Quality protein maize (QPM): Genetic basis and breeding perspective

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**Abstract:** Major fraction (60%) of seed storage protein in maize is zein which determines the quality of food and feed. Zeins comprise four subfamilies e.g.,  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  zeins. Among these,  $\alpha$ -zeins are the major prolamin subunits in maize.  $\alpha$ -zeins are rich in glutamine, leucine and proline but, deficient in essential amino acids like lysine and tryptophan causing malnutrition. The *opaque-2* (*o2*)-a natural recessive mutation in maize led to nearly double the lysine and tryptophan content in endosperm due to a decrease in the synthesis of zein proteins and increase in the other seed protein bound lysine and tryptophan. RNAi studies proved down regulation of 22kD zeins than the 19kD component as the biochemical basis of QPM phenotype. However, the *opaque-2* mutation made the endosperm chalky and soft resulting damaged kernel while harvesting, poor germination, increased susceptibility to pest and diseases, inferior for food processing and in general reduced yield. Later, combining *opaque-2* allele with its desirable genetic modifiers made it possible to breed QPM genotypes having hard kernel with high lysine and tryptophan content. Since, *opaque-2* is a recessive mutation and endosperm specific, and biochemical analysis of lysine and tryptophan content is expensive; conventional backcross breeding alone is inefficient for the nutritional enrichment of maize. However, use of *opaque-2* gene specific markers provided excellent opportunities for conversion of elite normal inbreds to homozygous *o2/o2* forms through marker assisted selection (MAS). In India, Vivek QPM-9: a hybrid of two QPM introgression lines is being widely used for commercial cultivation.

**Keywords:** Quality protein maize (QPM) - *Opaque-2* - Nutritional value - Introgression lines - QPM hybrids.

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### INTRODUCTION

Maize is the queen of cereal crops with highest grain yield potential. Endosperm is the store house of seed storage proteins. Maize grains contain around 9% protein. The major fraction (60%) of seed protein in maize is zeins (a prolamin group-alcohol soluble) (Leite *et al.* 1999) followed by glutelin (34%), while albumin and globulin occur in traces (3% each). A balanced protein is required to assist body building process and therefore, amino acid balance seems to be a determining factor for quality of any food and feed. Daily protein requirement for average Indian adult is 52 gm as against the availability of merely 26–30 gm in the daily diet. To alleviate malnutrition, protein content can be increased to as high as 18% by increasing the prolamine (zein) fraction in maize endosperm (Dudley & Lambert 1969), but unfortunately it consequently led to lysine and tryptophan deficiency. Many researchers around the globe have tried to address the problem using quality protein maize (QPM). In this pursuit, we focus on the genetic basis and prospects of quality protein maize for amino acid amelioration and enhancing the productivity of maize through the development of heterotic hybrids using elite QPM introgression lines.

### GENETIC BASIS

Zein seed proteins are the products of multigene families (Lending & Larkins 1989) and located within protein bodies on the rough endoplasmic reticulum (Larkins *et al.* 1993). Each of the zein polypeptide is a product of a differential structural gene (*Zp*). These *Zp* genes are simply inherited and are members of a large

group of genes (upto 150). Zein is particularly rich in glutamine (21–26%), leucine (20%), proline (10%) and alanine (10%), but deficient in important essential amino acids e.g., lysine and tryptophan leading to protein malnutrition.  $\alpha$ -zeins are the major prolamin subunits in maize, although other minor groups ( $\beta$ -15kD,  $\gamma$ -16 & 27kD and  $\sigma$ -10kD zeins) (Coleman & Larkins 1999, Leite *et al.* 1999) are also present in seeds. In normal maize,  $\alpha$ -zeins consist of two major sub-classes e.g., 19kD and 22kD zeins. Polymorphism arises from the presence of multigene families.  $\alpha$  and  $\sigma$ -zeins form the protein body core which is covered by peripheral  $\beta$  and  $\gamma$ -zeins (Lending *et al.* 1992, Esen & Stetler 1992).

The discovery of *opaque-2* (*o2*) natural mutants by Purdue university researchers paved the way for improving protein quality of maize endosperm protein (Mertz *et al.* 1964, Nelson *et al.* 1965). These mutants alter amino acid profile and composition of maize endosperm protein and result in two-fold tryptophan increase in the levels of lysine and tryptophan compared to normal maize. However, the pleiotropic effects of *opaque-2* mutation made the endosperm unpleasant taste, chalky, lighter and soft (starch granules loosely packed) resulting damaged kernel while harvesting, increased susceptibility to pest and diseases, inferior for food processing and in general reduced cob weight and lower yield due to reduced dry matter accumulation. For many years, this became the major hindrance in genetic improvement for higher productivity with enriched nutritional quality. Subsequently, the *opaque-2* mutation is reported to be associated with numerous modifiers which together behave as polygenic trait for kernel virtuosity. Later, concerted effort of Surinder K. Vasal and Evangelina Villegas (CIMMYT) made it possible to improve kernel hardness and grain yield by combining the *opaque-2* and its complex genetic modifier systems using modified backcrossing and recurrent selection. This gave birth to the quality protein maize (QPM).

There are several mutants that alter the amino acid profile of maize endosperm protein. These include *opaque-2*(*o2*), *floury-2* (*fl-2*), *Mucronate* (*Mc*) and *Defective endosperm B30* (*DEB30*). The *opaque* mutants are recessive (*o1*, *o2*, *o5*, *o9-11*, *o13*, *o16*, *o17*), the *floury* mutations are semi-dominant (*fl-1*, *fl-2* and *fl-3*) where as ‘*Mucronate*’ and ‘*Defective endosperm*’ are dominant mutations. The *opaque* mutations affect the regulatory network (Mertz *et al.* 1964, Nelson *et al.* 1965, Krivanek *et al.* 2007) whereas *floury*, *Mucronate* and *defective endosperm* affects the amino acid profile of storage proteins (Gibbon & Larkin 2005). Similar to *opaque 2*; *opaque-16* mutant allele is also reported to increase lysine and tryptophan content in the endosperm. Recently, a transposable element ‘*rbg*’ is reported to induce differential expression of *opaque-2* mutant gene in two *opaque 2* NILs derived from the same inbred line (Chen *et al.* 2014).

The molecular evolution of the *opaque-2* gene was investigated by Henry *et al.* (2005). Sequencing of most of the coding regions and parts of non-coding sequences of the *o2* gene in a set of cultivated and teosinte (wild progenitor of cultivated maize) accessions revealed 5.4% polymorphic sites and 72 insertions/deletions, located mostly in noncoding regions. Cultivated accessions retained about 70% of the diversity observed in teosintes. Besides, the molecular diversity in the *o2* transcriptional activator was reported to be quite high compared to that of other transcription factors in maize (Henry *et al.* 2005).

In fact, *opaque-2* (*o2*) is a natural recessive mutation in the transcriptional activator conditioning negative expression of zein protein (down regulation). This led to nearly double the lysine and tryptophan content in maize endosperm due to a decrease in the synthesis of zein proteins and increase in the other seed protein bound lysine and tryptophan (Henry *et al.* 2005). The *o2* gene has a large effect on lysine and protein content while minor on oil content (Lou *et al.* 2005). It seems that developing maize seeds possess compensatory mechanisms that sense protein content when zein synthesis is interrupted, leading to translation of other mRNAs instead of zein mRNAs. Combining deletion mutagenesis with current methods in genome and transcriptome profiling can make it possible to reveal alteration in amino acid composition (Holding 2014). RNA interference based down regulation of 22kD RNAi lines is reported to cause *opaque* phenotype more profoundly as compared to 19kD component. This is probably due to the greater interaction of 22 KDa components with  $\beta$  and  $\gamma$ -zeins resulting in a disruption in protein body formation which causes the *opaque* phenotype (Segal *et al.* 2003, Huang *et al.* 2004). Besides, Zhang *et al.* (2010) were able to trace the inheritance of *opaque-16* mutant allele which also linked to the elevated synthesis of lysine and tryptophan content. Transfer of this high lysine gene (*o16*) into *opaque -2* genetic background can further enhance the lysine content.

The details of proteome modulation that operates to alter amino acid composition are not clear. A microarray analysis of 1400 maize genes revealed that the 60 genes up-regulated more than three times in the mutant are related to stress responses, molecular chaperones and protein turn over (Prioul *et al.* 2008). Interestingly, among

the 66 down-regulated genes, many genes are involved in carbon, carbohydrate metabolism and branched chain amino acids (Hunter *et al.* 2002). Studies revealed that LKR/SDH (lysine-ketoglutarate reductase/saccharopine dehydrogenase) -the first enzyme of lysine catabolism is strongly depressed in o2 mutants as compared to wild type (Brochetto-Braga *et al.* 1992, Azevedo *et al.* 2003). During the process of evolution, segmental duplication in the progenitor genome of maize (Teosinte) is reported to result in diverged copies of the regulatory opaque-2 gene (Xu & Messing 2008). Recently, a 15.26kb duplication segment (qy27) at the 27-kDa  $\gamma$ -zein locus is reported to be a major modifier QTL which confers enhanced expression of this protein and leads to endosperm hardness (Liu *et al.* 2016). They mapped the said major QTL within a narrow interval of 100kb between the marker 0916-2 and Ch7-120.35 through fine mapping. Proteomic analysis using SDS-PAGE indicated that o2 introgression decreased the accumulation of various zein proteins except for 27-kDa  $\gamma$ -zein and also affected other endosperm proteins related to amino acid biosynthesis, starch-protein balance, stress response and signal transduction (Zhang *et al.* 2015, Zhou *et al.* 2016).

Pleiotropic effects of *opaque-2* gene has been recognized. Expression of the o2 gene environment labile and also depends on background polygenes which accounts for appreciable variation in amino acid composition and opaque phenotypes (Lou *et al.* 2005). However, In addition to alteration in amino acid composition, it affects starch organization making the kernel softer, opaque in appearance and unpleasant taste. A set of modifier genes (QTLs) in the opaque -2 genetic background are known to improve hard and vitreous kernels (Bjarnason *et al.* 1976, Ortega & Bates 1983, Burnett & Larkins 1999). Inheritance of o2 modifiers is complex (Vasal *et al.* 1980). Two major QTLs associated with endosperm modification have been identified. Vasal *et al.* (1980) combined the opaque-2 allele with QTLs for genetic modifiers and produced elite germplasm with the hard kernel and much higher quantity of lysine and tryptophan. The modified *opaque-2* mutants are reported to bring about reduced levels of 22 KDa  $\alpha$ -zeins and 2–3 times higher levels of 27 KDa  $\gamma$ -zeins (Geetha *et al.* 1991) along with increased hardness of the kernel (Moro *et al.* 1995). Besides, Krivanek *et al.* (2007) reported the involvement of a series of amino acid modifier genes for improvement of lysine and tryptophan. The genetics of amino acid modifiers is not well understood. Nevertheless, accumulation of favourable amino acid modifiers in o2 genetic background was accomplished by recombination breeding, and subsequently, several QPM pools were generated to provide genetic stocks for QPM breeding programmes (Sofi *et al.* 2009).

The opaque-2 gene was identified near to defective endosperm gene ‘DEB 30’ in the short arm of Chromosome 7 (Holding & Larkins 2008, Holding *et al.* 2008, Sofi *et al.* 2009); while, following RFLP analysis in an F<sub>2</sub> population, two major QTLs associated with endosperm modification have been identified near the centromere and telomere, respectively, on the long arm of the same chromosome (Lopes *et al.* 1995). A large number of reports followed wherein varying degrees of endosperm modifications were observed (Paez *et al.* 1969). The mechanism underlying the change of grain structure from chalky to vitreous in *modified opaque-2* mutants (mo2) is yet to be elucidated. But, breeders can undertake selection approach to accumulate QTLs to regulate this tightly controlled program (Dudley & Lambert 2004). Vasal *et al.* (1980) combined the *opaque-2* allele with QTLs for genetic modifiers and produced modified version of QPM germplasm with the hard kernel and much higher quantity of lysine and tryptophan.

A number of researchers revealed polymorphic molecular profiles of QPM lines using RAPD (Nkongolo *et al.* 2011, Hemavathy 2015), ISSR (Nkongolo *et al.* 2011, Lenka *et al.* 2015), SSR (Bantte & Prasanna 2003) and SNP (Semagn *et al.* 2012) markers. Bantte & Prasanna (2003) identified a few SSR markers e.g., bnlg 105, bnlg 125, bnlg 439, phi 037 and dupssr 34 with high polymorphic information content to differentiate QPM lines. But, none of these proved efficient to detect polymorphism between QPM and Normal maize inbreds. However, Identification of molecular markers that co-inherit with the *opaque 2* phenotype is a crucial step for their use in marker assisted breeding. CIMMYT designed o2-gene specific SSR primers viz., phi 057, phi 112 and umc 1066 which are located as internal repetitive elements within *opaque-2* gene on the short arm of chromosome 7 ([www.agron.missouri.edu](http://www.agron.missouri.edu)). Among these phi 057 and Umc 1066 are reported to be co-dominant while phi 112 is a dominant marker (Magulama & Sales 2009). Allelic polymorphism among QPM and normal maize inbreds was surveyed by several workers (Babu *et al.* 2005, Jompuk *et al.* 2006, Magulama & Sales 2009, Gupta *et al.* 2013) using these gene specific SSR primers and confirmed the codominant status of phi 057 and umc 1066. Phi 057 is reported to reveal a very good polymorphism with QPM donors showing a 169bp band and normal maize inbreds with a 159bp fragment (Magulama & Sales 2009). Bantte & Prasanna (2003) detected 30 unique SSR alleles to differentiate QPM inbreds. They also identified the SSR primer phi 057 to detect QPM

inbreds carrying opaque 2 mutation. Kata *et al.* (1994) used RFLP technique using Hind III digestion of genomic DNA and opaque-2 locus specific cDNA probe to detect o2/o2, o2/o2, and o2/o2 genotypes of individual plants in breeding populations. Besides, Zhang *et al.* (2010) reported the use of molecular marker umc1141 to trace the inheritance of the erstwhile mentioned opaque-16 mutant allele that led to the elevated synthesis of lysine and tryptophan content.

### **BREEDING FOR QUALITY PROTEIN MAIZE (QPM)**

Maize is a cross pollinated crop. The major breeding approach for increasing productivity is production of hybrids using heterosis breeding. The success of this method depends on development and identification of suitable inbred lines using an appropriate mating design; and selection of most promising heterotic normal maize hybrid. Now, QPM hybrids have been developed and tested for varying climatic and growing conditions. QPM varieties are grown on roughly 9 million acres worldwide following their spread from Mexico to throughout Latin America and to Africa, Europe, and Asia (Kataki & Babu 2003). For the production of QPM hybrid, the ultimate aim is to combine the advantage of heterosis along with amelioration of amino acid composition using QPM donors. To achieve this, conversion of both parental non-QPM inbreds to QPM status is the first step to develop a heterotic QPM hybrid. A number of reliable QPM donors (composite K; Ver 181-Ant gp venezuela-1, Thai composite, PD 9MS6 and composite-1) are now available at CIMMYT. These QPM donor stocks have been developed through intra-population selection for genetic modifiers in *opaque-2* backgrounds and are currently being used for large scale conversion of non-QPM inbreds to their QPM version. Subsequently, combining ability studies in QPM germplasm have been conducted and published (Vasal *et al.* 1993a, 1993b) so as to assist QPM hybrid development. Vivek *et al.* (2008) reported tryptophan content more than 0.60% and lysine content more than 2.6% in a set of CIMMYT QPM inbreds. Besides, Ortega & Villegas (1988) reported on an average 0.8% tryptophan and 3.1% lysine content among a set of QPM inbreds as against 0.4% and 1.6% respectively in normal maize lines. The inbred lines e.g., CML 176 and CML 186 are reported to be potential QPM donors (Manna *et al.* 2005, Danson *et al.* 2006) for introgression of o2 allele to non-QPM maize.

The opaque-2 allele is recessive (Vasal *et al.* 1993a, b) in nature, but the endosperm modifiers follow polygenic inheritance. At CIMMYT, a conservative approach is adapted to develop modified opaque 2 (mo2) genotypes to strike a balance between proteins levels and grain quality and competitive yield levels. However, molecular marker based screening for QPM status coupled with phenotypic selection to improve endosperm characteristics seems to be an appropriate strategy for the development of QPM introgression lines. The erstwhile mentioned gene-specific co-dominant markers phi 057 (Manna *et al.* 2005, Danson *et al.* 2006, Magulama & Sales 2009) and umc 1066 (Singh & Ram 2014, Gupta *et al.* 2009, Gupta *et al.* 2013) are worthwhile to trace the *opaque-2*(o2) allele in conversion programme. Distinct polymorphism revealed by both the primers can discriminate the QPM donors from respective non-QPM recurrent parents and also between homozygous (o2o2) and heterozygous (o2o2) *opaque-2* back cross progeny. This paves the way for rejection of non target BC progenies (dominant homozygous) resulting short cutting the breeding cycle (eliminates the need to grow F<sub>2</sub>) and substantial savings of labour and material resources for amino acid estimation (Tanksley *et al.* 1989, Frisch *et al.* 1999, Gupta *et al.* 2013). This made it possible to bred a QPM hybrid in less than half the time required in conventional breeding. Besides, foreground selection for opaque-2 combined with phenotypic selection for recipient parent at early back cross generations can bring about rapid recovery of recurrent parent genotype. The introgression lines developed using marker assisted back cross breeding may serve as important breeding material for the development of QPM hybrids.

Two Brazilian QPM varieties e.g., BR 451 and BR 473 developed by the Breeding Program of the National Maize and Sorghum Research Center (CNPMS - EMBRAPA) were released in 1988 and 1994 respectively for commercial cultivation. The former has been successfully used as a substitute for wheat due to its white color; while the yellow colour kernel of the later resembles normal maize resulting consumers acceptance as a substitute of normal maize. A mixture of wheat flour with that of BR 451 in a suitable proportion was reported ideal for industrial production of bread, cookies, and pasta (Peixoto *et al.* 1989). Besides, a double cross QPM hybrid was released in Brazil during 1997. Thereafter, a QPM hybrid Zhongdan 9407 was soon released in China. India has also released Shakti-1 in 1998 and is deeply involved in testing hybrids from CIMMYT. In India, Vivek QPM-9: a hybrid of two QPM introgression lines was released in 2008 (Gupta *et al.* 2009, Gupta *et al.* 2013) for commercial cultivation. CML 180 and CML 170 were selected as QPM donors for introgression of

*opaque-2* allele into the parental normal maize inbreds CM 212 and CM 145 through marker assisted backcross breeding. The said QPM hybrid, showed 41% increase in tryptophan and 30% increase in lysine over the original hybrid. However, the grain yield of the improved hybrid was on par with the original hybrid. Soon after, many countries participated in QPM network. The Republic of South Africa had earlier released hybrids HL-1, HL-2, and has recently released HL8 which has hard endosperm, good yield potential, and tolerance to diseases. In the similar line of work, marker-assisted backcross breeding (MABB) has been also used to develop maize genotypes for reduced anti-nutritional factors (Naidoo *et al.* 2012). Muthusamy *et al.* (2014) developed  $\beta$ -Carotene rich maize hybrids through marker-assisted introgression of  $\beta$ -carotene hydroxylase allele. Chander *et al.* (2008) identified a major locus (*y1*) for carotenoid content using gene targeted molecular marker (*Y1*ssr).

## CONCLUSION

Quality protein maize has a far-reaching impact on nutritional security with the discovery of *opaque-2* mutation. Such a natural recessive mutation led to selective down-regulation of specific zein genes resulting alteration in amino acid composition and opaque phenotype of endosperm. Modified marker assisted back cross breeding made it possible to develop QPM versions of normal maize inbreds with desirable endosperm characteristics and seed yield. These QPM introgression lines may be combined to develop QPM hybrids.

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