

Maize mutant *opaque2* and the improvement of protein quality through conventional and molecular approaches

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Abstract. Maize endosperm protein is deficient in two essential amino acids, lysine and tryptophan. Several spontaneous and induced mutations that affect amino acid composition in maize have been discovered amongst which the *opaque2* gene has been used in association with endosperm and amino acid modifier genes for developing quality protein maize (QPM), which contains almost double the amount of lysine and tryptophan compared to normal maize. These increases have been shown to have dramatic impacts on human and animal nutrition, growth and performance. A range of hard endosperm QPM germplasm has been developed at the International Maize and Wheat Improvement Center (CIMMYT) mostly through conventional breeding approaches to meet the requirements of various maize growing regions across the world. Microsatellite and SNP markers located within the *opaque2* gene provide opportunities for accelerating the pace of QPM conversion programs through marker-assisted selection (MAS). Thus, CIMMYT scientists are developing a package of reliable, easy to use markers for endosperm hardness and free amino acid content in the maize endosperm. Recent technological developments in molecular biology at CIMMYT such as single seed-based DNA extraction and low cost, high throughput SNP genotyping strategies promise enhanced efficiency and cost effectiveness of QPM molecular breeding programs. Here we present a summary of QPM research and breeding with respect to the history of conventional improvement methodologies, genetic and molecular basis of *opaque2*, epistasis between *opaque2* and other high lysine mutant genes and recent advances in genomics technologies that could potentially enhance the efficiency of QPM molecular breeding in future.

1. Introduction

Maize (*Zea mays* L.) plays a very important role in human and animal nutrition worldwide. In the mature maize kernel, two principal components, the endosperm and the germ (embryo), contain most of the kernel protein. Generally, the endosperm accounts for 80-85% and the embryo accounts for about 8-10% of the total kernel dry weight [1]. The endosperm may contribute as much as 80% of the total kernel protein. While the germ protein is superior in quality, the endosperm protein suffers from poor quality with respect to human and animal nutritional needs. The major drawbacks of maize endosperm protein are i) its deficiency in two essential amino acids – lysine and tryptophan, ii) high leucine – isoleucine ratio and iii) low biological value of utilizable nitrogen. The need to genetically ameliorate the poor nutritional value of maize has been recognized for long time and several mutations, both spontaneous and induced, have been identified that affect the amino acid composition of maize endosperm. Among them, a spontaneous mutation of maize with soft, opaque grains, named *opaque2* (*o2*), has been most intensively studied. Maize homozygous for the recessive *o2* allele has substantially higher lysine (>69%) and tryptophan content compared to normal maize [2].

1.1. Maize Protein

Maize endosperm protein is comprised of different fractions. Based on their solubility, these can be classified into albumins (water soluble), globulins (soluble in saline solution), zein or prolamine (soluble in alcohol) and glutelins (soluble in alkali). In normal maize endosperm, the average proportions of various fractions of protein are albumins 3%, globulin 3%, zein (prolamine) 60% and glutelin 34%, while the embryo protein is dominated by albumins (+60%), which are superior in terms of nutritional quality. The zein in maize endosperm is low in lysine content (0.1g/100g of protein), which negatively affects growth of animals [3]. In *opaque2* maize, the zein fraction is markedly reduced, by roughly 50%, with a concomitant increase in the relative amounts of nutritionally superior

fractions such as albumins, globulins and glutelins. The endosperm of *opaque2* maize contains twice as much lysine and tryptophan and 30% less leucine than normal maize. The decreased level of zein (5-27%) in *opaque2* maize along with reduced leucine, leads to more tryptophan for niacin synthesis and thus helps to combat pellagra and significantly improves its nutritional quality.

1.2. High lysine mutants in maize

Several mutants have been detected that favorably influence maize endosperm protein quality by elevating levels of two essential amino acids, lysine and tryptophan. The discovery of *opaque2* [2] was followed by recognition of the biochemical effects of *floury2* (*fl2*) [4]. Searches for new mutants continued and resulted in the discovery of several others such as *opaque7* (*o7*) [5], *opaque6* (*o6*) [6], *floury3* (*fl3*) [6], *mucronate* (*Mc*) [7] and *defective endosperm* (*De-B30*) [8]. Attempts were also made to find genotypes with high lysine genes that retained a high level of zein fraction. Two such mutants, *opaque7749* and *opaque7455* (*o11*) [9] are particularly interesting as they have markedly higher levels of lysine as well as a high prolamine fraction. The specific chromosomal location is known for some of the mutants. For example, the *o2* mutant is located on chromosome 7, *fl2* on chromosome 4, *o7* on chromosome 10, *fl3* on chromosome 8 and *de-B30* on chromosome 7. The genetic action of some of the mutants is also known, for example, *o2*, *o6*, *o7* and *o11* are completely recessive. The two floury mutants are semi-dominant and exhibit variable expression for kernel opacity and protein quality depending on the presence of one or more recessives in the triploid endosperm. The mutant *De-B30* is dominant and shows dosage effects on kernel opacity and zein content [10].

1.3. Pleiotropic and secondary effects of *opaque2* and other high lysine mutants

Genes and gene combinations that bring about drastic alterations in either plant or kernel characteristics also produce several secondary or undesirable effects. The low prolamine and high lysine mutants are no exception. In addition to influencing several biochemical traits, they adversely affect a whole array of agronomic and kernel characteristics. The *o2* and other mutants adversely affect dry matter accumulation resulting in lower grain yield due to increased endosperm size. The kernels dry slowly following physiological maturity of the grain and have a higher incidence of ear rots. Other changes generally associated with high lysine mutants include thicker pericarp, larger germ size, reduced cob weight, increased color intensity in yellow maize grains, and reduction in kernel weight and density. Thus, despite the nutritional superiority of *opaque2* maize, it did not become popular with farmers or consumers mainly because of reduced grain yield, chalky and dull kernel appearance and susceptibility to ear rots and stored grain pests. Hence, CIMMYT undertook to improve the phenotype of *opaque2* kernels to facilitate greater acceptability by developing hard endosperm grain types with the protein quality of chalky *opaque2* strains. CIMMYT received financial support, beginning in 1965, from the United Nations Development Program and introduced gene modifiers that changed the soft, starchy endosperm to a vitreous type preferred by farmers and consumers whilst retaining the elevated levels of lysine and tryptophan. CIMMYT has subsequently developed a range of hard endosperm *opaque2* genotypes with better protein quality through genetic selection, which are popularly known as quality protein maize (QPM). Today's QPM is essentially interchangeable with normal maize in both cultivation and agronomic characteristics as well as being competitive in terms of yield, lodging, disease and pest resistance, and moisture level, while retaining the superior lysine and tryptophan content. In 2005, QPM was planted on 695,200 hectares across 24 developing countries.

2. Conventional breeding approaches to develop QPM

There are various breeding options for developing hard endosperm - high lysine maize that is competitive in agronomic performance and market acceptance which are based on specific endosperm high lysine mutants or available donor materials. The past approaches involving normal maize breeding populations have centered on altering germ-endosperm ratio, selection for multiple aleurone layers, and recurrent selection to exploit natural variation for high lysine content. Altering the germ-endosperm ratio to favor selection of larger germ size will have the dual advantage of increasing both protein quantity and quality [11] but it is not practical to attain lysine levels approaching those of *opaque2* maize. Besides, increased germ size has the disadvantage of contributing to poor shelf life of

maize. Recurrent selection for high lysine in normal endosperm breeding populations has been largely unsuccessful due to the narrow genetic variation and heavy dependence on laboratory facilities of this approach. Alternatively, high lysine endosperm mutants provided two attractive options: i) exploiting double mutants involving *o2* and ii) simultaneous use of the *o2* gene with endosperm and amino acid modifier genes. In most instances, double mutant combinations involving *o2* and other mutants associated with endosperm quality were not vitreous [12]. The most successful and rewarding option exploited the combined use of *o2* with associated endosperm and amino acid modifier genes.

Segregation and analysis of kernels with a range of endosperm modification began at CIMMYT as early as in 1969 by John Lonnquist and V.L. Asnani. Initial efforts towards development of QPM donor stocks with good kernel phenotypes as well as good protein quality proved to be highly challenging. Two effective approaches, i.e., intra-population selection for genetic modifiers in *o2* backgrounds exhibiting a higher frequency of modified *o2* kernels and recombination of superior hard endosperm *o2* families, resulted in development of good quality QPM donor stocks with a high degree of endosperm modification. This was followed by the large-scale development of QPM germplasm with a wide range of genetic backgrounds, representing tropical, subtropical and highland maize germplasm and involving different maturities, grain color and texture. A summary of characteristics of promising QPM gene pools and populations developed at CIMMYT is provided in Table 1. An innovative breeding procedure designated as ‘modified backcross cum recurrent selection’ was designed to enable rapid and efficient conversion programs [13]. More recently, pedigree backcrossing schemes have been used to convert elite QPM lines to maize streak virus (MSV) resistance for deployment in Africa as well as conversion of elite African lines to QPM.

Table 1. Characteristics of QPM gene pools and populations developed at CIMMYT (using *o2* and associated modifiers) including protein, tryptophan and lysine contents in the whole grain [12]

QPM Pop/Pool	Adaptation	Maturity	Color	Texture	Protein (%)	Tryptophan in protein (%)	Lysine in protein (%)	Quality Index
Population 61	Tropical	Early	Y	Flint	9.2	0.98	4.2	3.8
Population 62	Tropical	Late	W	Semi-flint	9.9	0.92	3.9	4.4
Population 63	Tropical	Late	W	Dent	9.1	0.97	4.3	4.3
Population 64	Tropical	Late	W	Dent	9.6	1.00	3.8	4.3
Population 65	Tropical	Late	Y	Flint	9.2	0.96	4.2	4.4
Population 66	Tropical	Late	Y	Dent	9.3	1.01	4.3	4.3
Population 67	Subtropical	Medium	W	Flint	9.9	1.04	3.9	4.8
Population 68	Subtropical	Medium	W	Dent	9.5	1.01	4.0	4.3
Population 69	Subtropical	Medium	Y	Flint	10.0	0.98	4.2	4.4
Population 70	Subtropical	Medium	Y	Dent	9.3	1.10	4.3	4.7
Pool 15 QPM	Tropical	Early	W	Flint-Dent	9.1	0.94	4.2	4.6
Pool 17 QPM	Tropical	Early	Y	Flint	8.9	1.04	4.5	4.5
Pool 18 QPM	Tropical	Early	Y	Dent	9.9	0.93	4.0	4.6
Pool 23 QPM	Tropical	Late	W	Flint	9.1	1.03	3.8	4.2
Pool 24 QPM	Tropical	Late	W	Dent	9.4	0.92	3.8	4.0
Pool 25 QPM	Tropical	Late	Y	Flint	9.8	0.94	4.0	4.0
Pool 26 QPM	Tropical	Late	Y	Dent	9.5	0.90	4.1	4.3
Pool 27 QPM	Subtropical	Early	W	Flint-Dent	9.5	1.05	4.2	4.8
Pool 29 QPM	Subtropical	Early	Y	Flint-Dent	9.2	1.06	4.3	4.8
Pool 31 QPM	Subtropical	Medium	W	Flint	10.2	0.96	4.1	4.5
Pool 32 QPM	Subtropical	Medium	W	Dent	8.9	1.04	4.2	4.5
Pool 33 QPM	Subtropical	Medium	Y	Flint	9.3	1.05	-	4.2
Pool 34 QPM	Subtropical	Medium	Y	Dent	9.1	1.10	4.1	4.5

A QPM hybrid breeding program was initiated at CIMMYT in 1985 as the QPM hybrid product has several advantages over open pollinated QPM cultivars: a) higher yield potential comparable to the best normal hybrids, b) assured seed purity, c) more uniform and stable endosperm modification, and d) less monitoring of protein quality required during seed production. Several QPM hybrid

combinations were derived and tested through international trial series at multiple CIMMYT and NARS locations in Asia, Africa and Latin America. Current QPM breeding strategies at CIMMYT focus on pedigree breeding, whereby the best performing inbred lines and open pollinated varieties (OPV) with complementary traits are crossed to establish new segregating families. Both QPM×QPM and QPM × Normal crosses are made depending upon the specific requirements of the breeding project. In addition, backcross conversion is also followed to develop QPM versions of parental lines of popular hybrid cultivars that are widely grown in CIMMYT's target regions. Inbred lines developed through this process are then used in formation of QPM hybrids and QPM synthetic OPV [14].

3. Molecular basis of *o2* and modifier gene action

The breeding of QPM involves manipulation of three distinct genetic systems [14, 15]: i) the recessive mutant allele of the *o2* gene, ii) the endosperm hardness modifier genes and iii) the amino acid modifiers/genes influencing free amino acid content in the endosperm. The *o2* gene encodes a leucine-zipper class transcription factor that regulates the expression of zein genes and a gene encoding a ribosomal inactivating protein [16, 17, 18]. The homozygous recessive allele causes a decrease of the production of these zeins resulting in a corresponding increase in non-zein proteins, rich in lysine and tryptophan [19]. Additionally the recessive allele of the *o2* transcription factor also reduces the production of the enzyme, lysine keto-glutarate reductase, involved in free lysine degradation resulting in enhanced free lysine in the endosperm of *opaque2* maize. In the segregating generations, this recessive allele is selected either visually (identifying mosaic ears on F₂ harvests) or using molecular markers. The endosperm hardness modifier genes, which convert the soft/opaque endosperm to a hard/vitreous endosperm without much loss of protein quality, are selected through a low cost but effective method of light box screening, where light is projected through the vitreous grains or blocked by the opaque grains. Research at CIMMYT and elsewhere has demonstrated the quantitative and additive nature of the endosperm hardness modifying system [12]. Despite the presence of *o2* and associated endosperm hardness modifier genes, the lysine and tryptophan levels in segregating families vary widely indicating the existence of third set of genes that modify the amino acid content, which necessitates systematic biochemical evaluation of lysine and/or tryptophan levels in each breeding generation.

4. Molecular breeding for QPM

The *opaque2* gene is recessive and the modifiers are polygenic. Their introgression into elite inbred lines is not straight forward because of three major factors, i) each conventional backcross generation needs to be selfed to identify the *opaque2* recessive gene and a minimum of four to six backcross generations are required to recover satisfactory levels of the recurrent parent genome, ii) in addition to maintaining the homozygous *opaque2* gene, multiple endosperm modifiers must also be selected, and iii) rigorous biochemical tests to ensure enhanced lysine and tryptophan levels in the selected materials in each breeding generation require enormous labor, time and financial resources. Although conventional breeding procedures have been used to convert commercial lines to QPM forms, these procedures are tedious and time consuming. Rapid advances in genomics research and technologies has led to the use of MAS which holds promise in enhancing selection efficiency and expediting the development of new cultivars with higher yield potential [20, 21]. While marker-assisted foreground selection [22, 23] helps in identifying the gene of interest without extensive phenotypic assays, marker-assisted background selection [24, 25, 26, 27] significantly expedites the rate of genetic gain/recovery of recurrent parent genome in a backcross breeding program. With the development and access to reliable PCR-based allele-specific markers such as simple sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs), MAS is becoming an attractive option, particularly for oligogenic traits such as QPM [28].

A rapid line conversion strategy for QPM has been developed [29], consisting of a two-generation backcross (BC) program that employs foreground selection for the *opaque2* gene in both BC generations, background selection at non-target loci in the BC₂ generation, and phenotypic selection

for kernel modification and other desirable agronomic traits in two subsequent selfed generations. The rapid line conversion strategy outlined in this investigation brings together the salient features of both marker-assisted and phenotypic-based selection approaches such as fixing the large segregating generation for the target locus (*o2*), reducing the linkage drag by selection of flanking markers for recipient allele type, recovering maximum amount of recurrent parent genome within two BC generations and providing scope for precise phenotypic selection for desirable agronomic and biochemical traits on a reduced number of progeny.

4.1. Low cost marker for *o2* and reliable markers for modifier genes of QPM

SSR markers (*umc1066*, *phi057* and *phi112*) located within the *opaque2* gene provide an excellent foundation for MAS but this alone is not sufficient to bear the full effectiveness of molecular breeding for QPM genotypes. Each of the microsatellite markers located within the *o2* gene are associated with factors that challenge their routine use in MAS programs. *umc1066* is easily visualized on agarose gels but is commonly not polymorphic in CIMMYT breeding populations; *phi057* is difficult to visualize on agarose gels, usually requiring the use of polyacrylamide gels; *phi112* is a dominant marker and hence cannot be used in the identification of heterozygotes in F₂/BC populations. However, *phi112*, which is based on a deletion in the promoter region has the advantage of being a widely conserved marker, consistent with the phenotype in QPM germplasm tested. In order to overcome these difficulties, we have identified functional and more discriminative SNP markers that could be used in high throughput genotyping systems for selection of the *opaque2* genotype. These SNP markers have been used in the development of a medium throughput dotblot assay based on a detection system using hybridization on membranes, although these markers could also be readily detected using capillary electrophoresis systems.

Effective markers associated with modifying loci for both endosperm hardness and amino acid levels need to be identified. Unfortunately relatively little is known about the number, chromosomal location and mechanism of action of these modifier genes. A complex system of genetic control of these modifier loci with dosage effects, cytoplasmic effects, incomplete and unstable penetrance in different QPM germplasm creates a major bottleneck to the accelerated development of QPM germplasm. Using a limited set of restriction fragment length polymorphism (RFLP) markers and bulked segregant analysis [30], two chromosomal regions on the long arm of chromosome 7 that are associated with *o2* endosperm hardness modification were identified. The locus near the centromere is linked with the gene encoding the 27 kDa gamma zein. More recently, the analysis of two different QPM genotypes, K0326Y and CM105Mo2 (derived from CIMMYT's Pool 33 QPM) corroborated the existence of a common quantitative trait locus (QTL) near the centromere of chromosome 7 that appears to have a major effect (30% of the phenotypic variance) on *o2* endosperm modification, in addition to a QTL on 9.04/9.05 [31]. In a specific F₂ population segregating for kernel vitreousness, these two loci accounted for 40% of the phenotypic variation and thus may prove to be strong candidates for MAS for QPM breeding.

Precise information on genes controlling the level of amino acid modification especially with respect to lysine and tryptophan is relatively scarce and studies to date have found several quantitative trait loci (QTL) on many of the maize chromosomes [32, 33]. The free amino acid (FAA) content in Oh545o2 is 12 times greater than its wild-type counterpart, and three and 10 times greater than in Oh51Ao2 and W64Ao2, respectively. QTL mapping involving these materials identified four significant loci that account for about 46% of the phenotypic variance for FAA [34]. One locus on the long arm of chromosome 2 is coincident with genes encoding a monofunctional *aspartate kinase 2* (*Ask2*) whereas another locus on the short arm of chromosome 3 is linked with a cytosolic triose phosphate isomerase 4. Subsequent feedback inhibition analysis has suggested that *Ask2* is the candidate gene associated with the QTL on 2S [35] and that a single amino acid substitution in the C-terminal region of the *Ask2* allele of Oh545o2 is responsible for altered basal activity of the enzyme [36]. Using a RIL population from the cross between B73o2 (an *o2* conversion of B73) and a QPM line (CML161), it was possible to identify three QTL for lysine content and six QTL for tryptophan content, which explained 32.9% and 49.1% of the observed variation, respectively [37]. Thus a series of molecular markers (Table 2) for manipulation of different genetic components of QPM is available

and hence their validation and fine mapping in appropriate breeding populations should now be carried out in order to establish a single cost effective MAS assay for molecular breeding of QPM. Concerted research efforts to quantify the effect of these loci affecting endosperm hardness and amino acid levels coupled with marker development and validation will also accelerate the pace and precision of QPM development programs.

Table 2. Molecular markers currently being validated at CIMMYT for manipulation of different genetic components of QPM

Chromosome	Flanking markers	LRS*	PEV**	Reference
1) opaque2 gene				
7S	<i>umc1066</i> , <i>phi057</i> , <i>phi112</i> (located within the <i>o2</i> gene)			[29,38]
2) Endosperm harness modification (based on K0326YQPM × W64Ao2)				
1.05-1.06	<i>umc1076-umc1335</i>	12.8	5	[31]
7.02	<i>umc1978-bnlg1022</i> (27kDa <i>gamma zein</i> gene)	75.3	28	[31]
9.04-9.05	<i>umc1771-umc1231</i>	29.4	12	[31]
10.02	<i>phi063-umc1432</i>	16.7	11	[31]
3) Free amino acid content (FAA)				
2L	<i>bmc1633-bmc1329</i> (<i>aspartate kinase2</i>)	14.8	11	[34,36]
2S	<i>bmc1537-bmc2248</i>	12.8	10	[33]
3S	<i>bmc1904-bmc2136/bmc1452</i>	17.9	15	[33]
7L	<i>bmc2328b-phi045</i>	12.6	10	[33]

*LRS is the likelihood ratio statistic that measures the significance of the QTL. **PEV (percent explained variance) is the percent of total variance explained by the QTL.

4.2. Seed DNA-based genotyping and MAS for QPM

Leaf collection from the field, labeling and tracking back to the source plants after genotyping are rate limiting steps in leaf DNA-based genotyping. Recently, an optimized genotyping method using endosperm DNA sampled from single maize seeds was developed at CIMMYT [39], which has the potential to replace leaf DNA-based genotyping for marker-assisted QPM breeding. This method is suitable for various types of maize seeds, produces high quality and quantity of DNA and has minimal effects on subsequent germination and establishment. A substantial advantage of this approach is that it can be used to select desirable genotypes before planting, which can bring about dramatic enhancements in efficiency by planting only the plants containing *o2* gene in recessive form in BC_nF₂ generations of Normal × QPM crosses, and also by minimizing the labor costs and scoring error associated with light-box screening of a large number of grains for endosperm hardness. Over several breeding cycles, this is likely to lead to cumulative and accelerated gains in selection pressure (such as light box screening for endosperm hardness modification and systematic biochemical evaluation of lysine and/or tryptophan) and improvements in overall QPM breeding efficiency.

4.3. Integrated approaches for improvement of enhanced protein quality in maize

Research at CIMMYT is currently focused on developing a package of molecular markers for cost-effective large scale, marker-assisted QPM breeding program. We have developed gene-based SNP markers and a medium throughput, low cost dot blot genotyping assay using a membrane-based hybridization system for the *opaque2* gene. This system is likely to be especially useful for NARS programs with limited lab facilities. In addition, intensive efforts are being made to develop and validate new as well as existing markers for the endosperm and amino acid modifier genes across a wide range of populations and improved pools. A new breeding program has recently been initiated at CIMMYT in collaboration with Guizhou Academy of Agricultural Sciences, China, to pyramid the *o2* with another high lysine mutant, *o16*, which was selected from Robertson's *Mutator* (*Mu*) stock where the lysine content in the F₃ *o2o2o16o16* families derived from recombination of both *o2* and *o16*, was about 30% higher than that of *o2o2* or *o16o16* F₃ families [40]. Using both genes together could lessen the requirement for phenotypic screening for amino acid content and genetic screening for amino acid modifiers.

Recent efforts in genetic transformation are focused on developing a dominant *opaque2* trait in maize. RNA interference (RNAi) technology has been used to reduce 22-kDa [41] and 19-kDa alpha zeins

[42, 43] using antisense transformation constructs, which result in moderate increases (15-20%) in lysine content. In a recent study, using an improved double strand RNA (dsRNA) suppression construct, Huang et al. [44] reported lysine and tryptophan levels similar to conventionally bred QPM genotypes. While the dominant nature of the anti-sense transgene is a definite advantage as compared to recessive allele of *o2*, the opaque endosperm still needs to be modified by endosperm modifier genes whose epistasis with the transgene has not yet been tested. In addition, social acceptance and legislative concerns regarding genetically modified crops may prove to be a major bottleneck to their practical utility and large scale adoption in some developing countries. Efforts are underway at CIMMYT-Africa to create transgenic events using RNAi that could potentially enhance lysine and tryptophan to levels similar to QPM. Identification and improvement of lines with enhanced regenerability and transformability is in progress to facilitate RNAi efforts. Tropical maize lines, CML390, CML442, CML254 and CML492 were identified for their better regenerability and transformability, while CML395 was improved for transformation efficiency by crossing to temperate inbred line A188.

4.4. Cost effectiveness of marker assisted QPM breeding programs

Detailed cost-benefit analysis carried out at CIMMYT [45] with respect to MAS for QPM (*o2* gene alone) suggested that the relative cost effectiveness would depend upon specific circumstances. In cases where segregating materials can be visually inspected or light box screened to identify *o2* containing ears, conventional methods may prove to be more cost-effective although less accurate, while MAS may be a valuable tool with certain genetic backgrounds which do not allow easy phenotypic detection of *o2*. More recently, four BC₁F₂ populations segregating for *o2* were used to test the error rate of light-box screening and to estimate the cost of genotyping using a seed DNA-based genotyping method recently developed at CIMMYT which makes it possible for MAS to be carried out before planting. For two populations where the light-box error rate was over 30%, it is predicted that there is an overall benefit from using MAS. Effective use of molecular markers for QPM would be achieved through seed DNA-based genotyping, and use of flanking markers around the *o2* gene to improve the efficiency of backcross selection. In addition, simultaneous MAS for the *o2* gene as well as modifier genes for amino acid content and endosperm hardness would provide a much more compelling cost-benefit ratio. This would reduce the need for phenotypic screening, saving time and screening costs. Moreover, when MAS is implemented for simultaneous selection of endosperm modifiers and other multiple traits such as disease resistance and other quality traits, the added value of MAS will be cumulative in terms of cost and time efficiency as well as selective gain.

5. Conclusion

Biofortification of maize grains is an important area of research for which *opaque2* provides an ideal platform upon which a number of nutritionally important traits such as enhanced Fe and Zn content and low phytate content (for increased bioavailability of nutrients) could be combined for multiple benefits. Considering the pace of technological developments in genome research, a molecular breeding option is likely to be the leading choice in the future for stacking a range of nutritionally important specialty traits, especially those governed by recessive genes. With respect to *opaque2*, an additional challenge to field implementation of QPM is its recessive nature [14]. If QPM is pollinated by normal maize pollen, there may be loss of high protein quality resulting in erosion of the trait in farmer saved seed systems. Though several years of QPM testing at CIMMYT and elsewhere has proved this apprehension to be not significantly valid, training on good seed production practices to the local communities may ensure sustainable higher nutritional benefits of QPM in the long term.

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