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RESEARCH ARTICLE

Genetic enhancement of essential amino acids for nutritional enrichment of maize protein quality through marker assisted selection

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Abstract Maize grain protein is deficient in two essential amino acids, lysine and tryptophan, defining it as of low nutritive value. The discovery of *opaque2* (*o*2) gene has led to the development of quality protein maize (QPM) that has enhanced levels of essential amino acids over normal maize. However, the adoption of QPM is still very limited. The present study aims at improving the quality of normal four maize inbred lines (LM11, LM12, LM13 and LM14) of single cross hybrids; Buland (LM11 × LM12) and PMH1 (LM13 × LM14) released in India for different agro-climatic zones by introgressing *o*2 allele along-with modifiers using marker assisted backcross breeding. Both foreground and background selection coupled with phenotypic selection were employed for selection of *o2* specific allele and maximum recovery of the recurrent parent genome (87–90%) with minimum linkage drag across the crosses. The converted QPM lines had < 25% opaqueness which is close to the respective recurrent parents. The QPM versions showed high level of tryptophan content ranging from 0.72 to 1.03 across the four crosses. The newly developed best QPM lines were crossed in original combinations to generate QPM hybrids. The grain yield of improved QPM hybrids was at par and there was significant increase in tryptophan content over the original hybrids.The integrated marker assisted, and phenotypic selection approach holds promise to tackle complex genetics of QPM. The dissemination and adoption of improved QPM versions may help to counteract protein-energy malnutrition in developing countries.

Keyword Biofortification · Introgression · Marker assisted selection · Nutritional value · *Opaque2* gene · Tryptophan content · *Zea mays*

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Introduction

Maize (*Zea mays* L.) is the third most important cereal crop that contributes 15% of the proteins and 20% of the calories derived from food crops of the world's diet. It is considered as nutri-cereal due to the fact of high content of carbohydrates, fats, proteins and some of the important vitamins and minerals. In the developing countries people fulfill their protein and calorie requirements from maize. But unlike other cereals, it is deficient in lysine and tryptophan which are essential amino acids, thus making it inadequate in nutritional quality (Nelson 1969). The breakthrough for genetic improvement of protein quality commenced with the discovery of high lysine mutant *opaque2* (*o2*) (Mertz et al. 1964) and later on another mutant *floury2* (*fl2*) (Nelson et al. 1965) followed by other high lysine mutants such as *o6*, *o7*, *o9*, *o11*, *o13*, *fl1*, *fl3*, *De*-B30*, *Mc*, *Sh2*, and *bt2* (Vasal 2002). It has been reported that regulatory genes are affected by recessive mutations while semi-dominant and dominant mutations affect the storage proteins (Vikal and Chawla 2014). All these mutations resulted in increase of lysine and tryptophan due to increase in non-zein protein content and

decrease in the level of α-zeins (Damerval and Vienne 1993; Habbenet al. 1993). Hence the mutants have higher nutritional and biological value. But exploitation of these mutant genes except *o2* was stuck due to mortality of the mutant seedlings and the complex genetics of high lysine trait (McWhirter 1971; Nelson 1979, 1981).

The *O2* gene located on chromosome 7 encodes basic leucine zipper (*bZIP*) transcriptional regulator that modulates expression of 35 target genes by binding to ACGT core sequence in its target genes as revealed by RNA-seq and CHIP-seq approach (Li et al. 2015a, b). The *O2* gene activates the expression of 22-kDa α-zein and 15-kDa β-zein genes (Schmidt et al. 1990). *O2* also regulates other non-storage protein genes such as *b-32*, encoding a type I ribosome-inactivating protein, one of the two cytosolic isoforms of the *pyruvate orthophosphate dikinase* gene (*cyPPDK1*), *starch synthase III* gene and *b-70*, encoding a heat shock protein 70 analogue, possibly acting as a chaperon during protein bodies' formation. The mutated *o2* gene reduces the level of 22 kDa α-zein and enhances the synthesis of a number of non-zein proteins, especially, EF-1α which is positively correlated with lysine content in the endosperm (Damerval and Vienne 1993; Habben et al. 1993; Gaziolaet al. 1999). The *O2* gene is also known to be involved in synthesis of enzyme lysine-ketoglutarate reductase that is associated with free lysine degradation. As a consequence, in the grain with *o2* mutation, a dramatic reduction in this enzyme leads to a corresponding increase in free lysine in the endosperm (Kemper et al. 1999) and also inhibits a lysine-sensitive aspartate kinase isoenzyme of the aspartate biosynthetic pathway (Azevedo and Arruda 2010). *O2* itself is positively autoregulated by its gene product due to the presence of B5-like box in its promoter region (Rossi et al. 1997).

However, the *o2* gene was associated with many undesirable pleiotropic effects, such as slow drying, low grain yield, opaque endosperm, more vulnerability to ear rots and storage pests (Bajarnason and Vasal 1992; Prasanna et al. 2001). As a result, the manipulation of *o2* mutant and its extensive acceptance in breeding programmes was hampered (Ortega and Bates 1983; Lauderdale 2000; Krivanek et al. 2007). Overtime, the *o2* related limitations had been overcome by subsequent selection of modifier genes using conventional breeding methodologies by breeders at International Maize and Wheat Improvement Center (CIMMYT), Mexico. The improved material restored the desired hard endosperm in the genetic background of recessive *o2* mutation. The increased level of γ-zein by modifiers had contributed to the recovery of hard endosperm (Wallace et al. 1990). The *o2* genotypes with endosperm modifiers, having elevated lysine and tryptophan levels, were designated as 'Quality Protein Maize' (QPM) (Vasal et al. 1980; Bajarnason and Vasal 1992; Geevers and Lake 1992). The nutritional value of QPM has enhanced which made it commercially acceptable. The modified *o2* version maize lines have varying levels of lysine and tryptophan content due to the presence of amino acid modifiers. The complementation of amino acid modifiers from both parents is responsible for the significant increase of lysine level in the seed (Krivanek et al. 2007). This necessitates systematic biochemical evaluation of lysine and tryptophan levels. As most biofortification efforts are still on the way, the development and dissemination of QPM provides a valuable source for securing the nutritional value of maize.

The introgression of *o2* gene into normal inbreds is not a straightforward procedure due to its recessive nature and each conventional backcross generation needs to be selfed to identify the *o2* gene. On the other hand, modifiers are polygenic so at least six backcrosses are required to recover satisfactory levels of the recurrent parent genome along with hard endosperm and high levels of essential amino acids. Long time back the tedious and time-consuming conventional breeding efforts have been used to convert commercial lines to QPM versions (Vasal 2000). The development of DNA marker technology has led to the use of DNA marker assisted selection (MAS) that accelerated the selection efficacy and expedited the generation of new cultivars with higher yield potential (Babu et al. 2005). Breeding for QPM genotype becomes speedy with the use of *o2* locus specific simple sequence repeat (SSR) markers which allowed breeders to discard non-QPM plants prior to pollination and selection of either heterozygous or homozygous genotypes for *o2* during marker assisted backcross breeding. Thus, the present study aims to incorporate *o2* gene into four non-QPM parental inbred lines (LM11, LM12, LM13 and LM14) of two maize hybrids, Buland (LM11 × LM12) and PMH 1 (LM13 × LM14), and reconstitution of the QPM version of these hybrids using an integrated approach of marker assisted and phenotypic selection. Both Buland and PMH1 hybrids are high yielding and recommended for cultivation during winter and monsoon season, respectively in Punjab and at National level with an average yield of 7750 and 5500 kg/ha, respectively. The reconstituted QPM hybrids had enhanced levels of tryptophan compared to original hybrids. The implementation of developed QPM versions may help in providing a nation's nutritional security.

Materials and methods

Plant material

Four non-QPM maize inbreds namely LM11, LM12, LM13 and LM14 were used as recurrent parents. These lines are

parental lines of two maize hybrids, Buland (LM11 × LM12) and PMH 1 (LM13 × LM14). The inbreds DMR7, DMR56, CML161, CML162, CML163, CML165, and CML170 were used as QPM donor pollen parent. Only few crosses were carried forward to generate backcross progenies.

Generation of backcross progenies

Marker assisted backcross breeding (MABB) was followed for introgression of *o2* gene in the background of four recurrent parents following Babu et al. (2005) methodology. The F_1 was made using non-QPM parent as female and QPM as the pollen parent in different cross combination enlisted in Table S1. The crosses were attempted using both DMR and CIMMYT QPM sources to combine the two parents of a hybrid with different kernel modifiers. Based on genetic diversity analysis (Vikal et al. 2013), few crosses were carried forward to generate backcross populations viz. LM11 × CML170, LM12 × DMR7, LM13 × CML165 and LM14 × CML162. F_1s hybridity was confirmed using *o2* gene specific marker. True F_1s were backcrossed twice to develop BC_1F_1 and BC_2F_1 progenies with respective crosses. The BC population size comprised of 109–262 seeds per cross. Selected BC_2F_1 plants were self-pollinated to produce BC_2F_2 population of each cross. The homozygous BC_2F_2 plants for *o2* allele were selected and selfed to generate BC_2F_3 progenies of each cross. The BC_2F_3 progenies were raised in ear to row manner and evaluated for agronomic characters. The best recovered plants for recipient genome were selfed to generate BC_2F_4 progenies. The schematic representation of MABB is given in Fig. 1.

Foreground and background selection

Three SSR markers (*phi057*, *phi112* and *umc1066*) present within the *o2* gene enabled direct selection for the *o2* gene without false positive and false negative in breeding programs (Babu et al. 2005). The *phi112* SSR is located between the G box and three upstream open reading frames (u ORF's) in the leader sequence of the *o2* gene. The *umc1066* and *phi057* SSRs are located in exon 1 and exon 6, respectively (Yang et al. 2004). Two *o2* gene-based markers viz. *phi057* and *umc1066* were employed for foreground selection of heterozygotes in each backcross generation and homozygotes in selfed generation. SSR marker *umc1066* was used in LM11 and LM13 crosses whereas *phi057* was surveyed in LM12 and LM14 crosses. The segregation of the marker locus in each generation was authenticated for the expected Mendelian ratio by using standard Chi square test.

A total of 274 SSR (simple sequence repeat) markers spanning all bins of 10 linkage groups (https://www.maizegdb.org) were surveyed for parental polymorphism and polymorphic SSR markers were analyzed for background selection on DNA of BC_2F_1 plants for selection of plants with maximum recurrent parent genome (RPG) recovery in each cross. The percentage recovery of the RPG was calculated as the ratio of number of homozygous SSRs for the recurrent parent to the total number of polymorphic SSRs.

Genotyping

Genomic DNA was extracted from young leaves of F_1, BC_1F_1, BC_2F_1 and BC_2F_2 seedlings using the modified CTAB (cetryl trimethyl ammonium bromide) method (Saghai-Maroofet al. 1984). In vitro amplification was performed in a 96 well microtiter plate in Veritti 96 well (Applied Biosciences, Invitrogen, UK) and Gene Amp® PCR system 9700 (Applied Biosystems by Thermofisher scientific) master cyclers. The reaction mix for each 20 µl reaction volume had 100 ng template DNA, 100 µM of each dNTP, 1.5 mM $MgCl_2$, 1X PCR reaction buffer (10 mM Tris/HCl + 50 mM KCl + 0.01% w/v gelatin, pH 8.3), 0.5 µM each of forward and reverse primers and 1.0 unit of Taq polymerase. The amplification was achieved using thermal profile of initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 50–65 °C (depending on the primer) for 2 min and extension at 72 °C for 2 min, and final extension at 72 °C for 10 min. The amplified product was kept on hold at 4 °C after the completion of thermal profile. A negative control (without template DNA) was included in each plate during every amplification reaction. The PCR products were resolved on 3% agarose gel at 5 V/cm for 3–5 h. The amplicons were visualized under UV transilluminator and documented in the gel documentation system (UVP, USA).

Phenotypic selection for kernel modification

Kernels from BC_2F_2 ears segregated for varying degree of softness and hardness in all the crosses. Kernels were kept on light box table and white light was projected through the grains for visual categorization of different types of endosperm. Based on their opaqueness and graduation in the opaqueness, kernels were scored on 1 to 5 scales. Score 1 is given for fully modified kernels (0% opaqueness), score 2, 3, 4 for 25%, 50%, 75% opaqueness, respectively, whereas score 5 for soft, chalky 100% opaque kernels. The BC_2F_3 seeds with $< 25\%$ opaqueness was selected for seed multiplication in each cross.

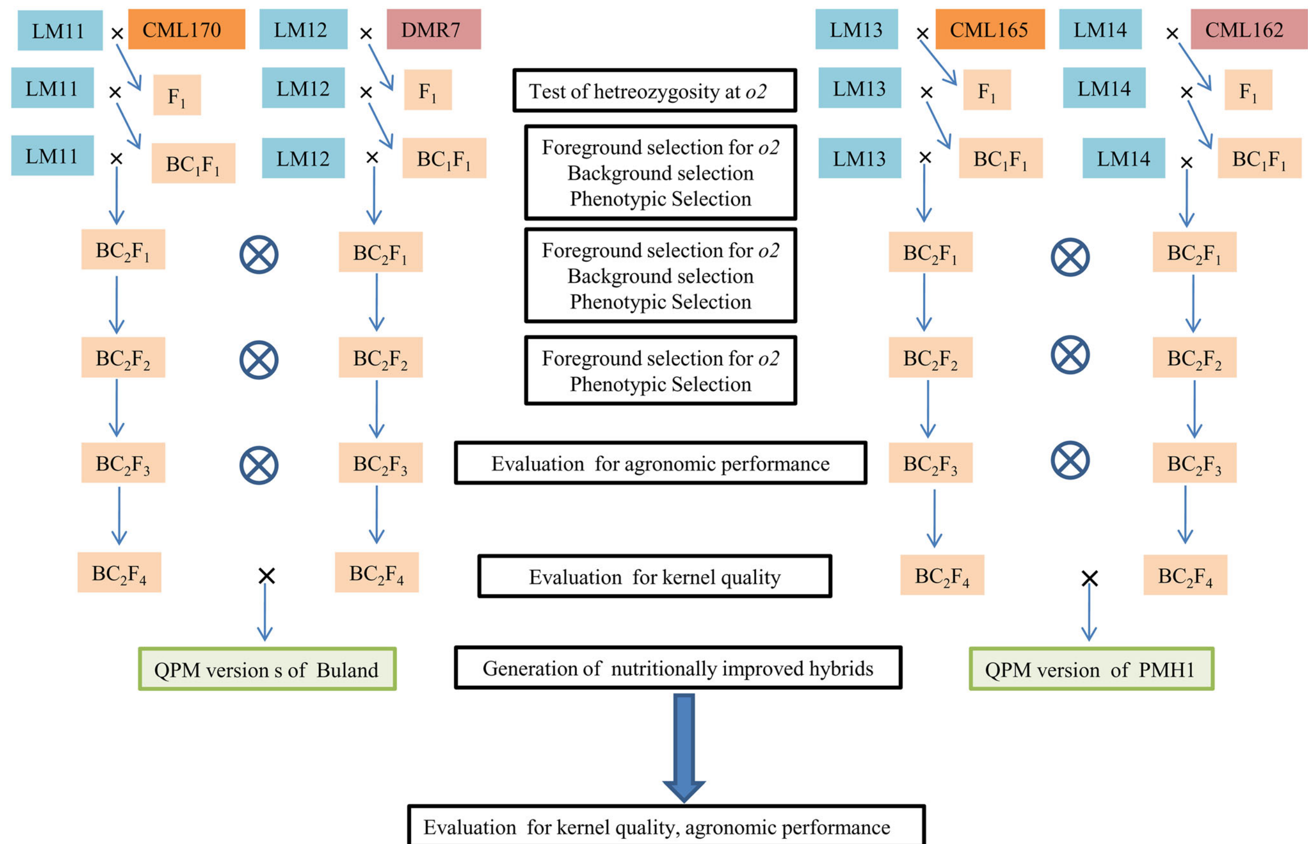

Fig. 1 A schematic representation of simultaneous conversion of four normal inbreds to QPM versions and development of QPM hybrids

Biochemical analysis

Twenty grains from each BC_2F_4 ear were grinded into powder and dried at 80 °C for 2 h. The protein content was determined using Infratec 1241 Grain Analyzer. Hernandez and Bates (1969) observed that the relationship between tryptophan and lysine in the maize endosperm protein is about 1 to 4; thus, tryptophan estimation was used as a single parameter for maize quality evaluation. Tryptophan content was analyzed by spectrophotometer (Villegas and Mertz 1971). Tryptophan content in percentage was calculated as Quality Index (QI). The quality index is the tryptophan to protein ratio in the sample, expressed as a percentage. QI = x*0.004*100/total protein, where x is reading at OD545. Total protein and tryptophan of the seed samples were estimated in technical triplicates. The data generated for tryptophan content was analyzed to calculate the standard error of the mean.

Evaluation for agronomic traits

The BC_2F_3 progenies of all the crosses were evaluated for various agronomic characters such as plant height, days to anthesis, days to silking, tassel density, tassel length, anther glume color, anther color, silk color, ear length and kernel color. Each progeny was raised in two rows of 3 m row length with a plant to plant distance of 20 cm and row to row distance of 60 cm. The plant height (cm) was taken from the base of plant to node bearing tassel. Anther glume color was marked from green to pink. Anther color was recorded between cream and pink. Days from planting to anthesis and silking, were documented when 50% of plants had extruded anther or produced silk. Data was recorded on daily visual observation during the flowering period. Tassel length (cm) was recorded from node bearing the tassel to the tip of the tassel. Tassel density was recorded from low to high and kernel color from yellow to deep orange.

Reconstitution of QPM hybrids

The stable uniform BC_2F_4 converted QPM versions were crossed in original parental combinations to reconstitute the original QPM hybrids. The original hybrids and their improved versions along-with QPM released checks were evaluated in three replications with two-rows/entry at the Research Farm of Punjab Agricultural University, Ludhiana and Regional Research Station, Gurdaspur during winter season (Buland hybrid versions) and summer season

(PMH 1 hybrid versions). Standard agronomic practices were followed for raising the crop. Data was recorded on grain yield and quality parameters.

Results

Parental polymorphism

The parental lines were validated for the polymorphism using gene based SSR markers of *o2* gene. The SSR marker *umc1066* showed polymorphism between the recurrent parents—LM11, LM13 and donor parents—CML162, CML165 and CML170. The amplified allele was of 160 bp size in LM11and LM13. The amplicon of 145 bp was amplified in donor lines namely CML162, CML165 and CML170. The SSR marker *phi057* was polymorphic between LM12, LM14, DMR7 and CML162 and amplified approx. The 140 and 150 bp fragment in recurrent parents, LM12 & LM14, respectively whereas 170 bp fragment was amplified in QPM inbred lines, DMR7 and CML162, respectively (Fig. S1). Thus, the SSR marker *phi057* was used for foreground selection of *o2* locus in the crosses involving LM12 & LM14 as recurrent parent whereas SSR marker *umc1066* was employed in crosses involving LM11 & LM13 as the recurrent parent.

A total of 264 SSR markers were surveyed among the non-QPM and QPM lines for parental polymorphism. Overall, 103 SSR markers were polymorphic between LM11 and CML170; LM12 and DMR7 while 104 and 107 SSR markers showed polymorphism between LM13 and CML165 and LM14 and CML162, respectively (Table 1). The amplification pattern of few SSR markers used for parental polymorphism survey is shown in Fig. S2.These identified polymorphic markers were used for background selection.

Foreground selection for *o2* gene in BC_1F_1 and BC_2F_1 generation

A total of 148, 125, 210 and 109 BC_1F_1 plants were subjected to foreground selection for *o2* allele in crosses viz. LM11/CML170/1*LM11, LM12/DMR7/1*LM12, LM13/CML165/1*LM13 and LM14/CML162/1*LM14, respectively. The number of heterozygotes for the *o2* locus was 67, 56, 115 and 46 in each cross of LM11/CML170/1*LM11, LM12/DMR7/1*LM12, LM13/CML165/1*LM13 and LM14/CML162/1*LM14, respectively. The heterozygous BC_1F_1 plants of each cross were visually analyzed for recurrent parent specific traits like silk color, anther color, tassel shape, tassel density. The 20–25 visually selected plants were again backcrossed to the respective recurrent parent of each cross to generate BC_2F_1 progenies. Of the 220, 188, 206 and 262 plants in BC_2F_1 of LM11/CML170//2*LM11, LM12/DMR7//2*LM12, LM13/CML165//2*LM13 and LM14/CML162//2*LM14; 100, 87, 114 and 120 plants were heterozygous for *o2* locus, respectively. The marker segregated according to the expected Mendelian ratio of 1:1 in each BC generation (Table 2).

Background selection for recurrent parent genome in BC_2F_1

The heterozygous progenies for *o2* locus were subjected to background screening using polymorphic SSR markers. There was a wide range of variation for recurrent parent genome recovery among the progenies of each cross. Overall, the recipient genome content varied from 57 to 90% across the four crosses. The recovery of the recipient genome in the best five BC_2F_1 plants ranged from 82.1% to 87.3% for LM11/CML170//2*LM11 cross, whereas it was 85% to 89.3% for LM12/DMR7//2*LM12 cross (Table 1). Similarly, recipient genome content in the best five BC_2F_1 plants of cross LM13/CML165//2*LM13 ranged from 87.3% to 90.4% and it was 86.4% to 89.7% for cross LM14/CML162//2*LM14. Nine, thirteen, ten and fifteen BC_2F_1 plants of LM11/CML170//2*LM11, LM12/DMR7//2*LM12, LM13/CML165//2*LM13 and LM14/CML162//2*LM14 respectively, were selected on the basis of RPG recovery and visual phenotypic assessment for selfing to develop BC_2F_2 progenies. Based on BC_2F_2 cob shape and kernel color, three ears from each cross were chosen for developing BC_2F_2 population to fix the *o2* allele.

Foreground selection for *o2* gene in BC_2F_2 generation

Foreground selection was employed for selecting *o2* gene in homozygous recessive form among BC_2F_2 populations. In cross LM11/CML170//2*LM11 and LM14/CML162//2*LM14 out of 150 and 160 plants analyzed, 42 and 37 plants were homozygous recessive for the *o2* gene, respectively. Out of 200 plants of cross LM12/DMR7//2*LM12, 48 plants were homozygous dominant, 96 were heterozygous and 56 were homozygous recessive for the *o2* gene. Similarly, for cross LM13/CML165//2*LM13 out of total 194 plants screened 54 plants were homozygous dominant, 101 were heterozygous and 39 were homozygous recessive for the *o2* gene (Fig. 2). The data on foreground selection of *o2* gene is summarized in Table 2. Stringent phenotypic selection was made and 10 plants from each cross were selfed to fix the *o2* allele in the homozygous recessive state.

Table 1 List of simple sequence repeats (SSR) markers analyzed and polymorphic SSR markers used in the BC_2F_1 families and the recovery of the recurrent parent genome (RPG) in the best lines

Chr. No.[a]	Number of markers analyzed	Number of polymorphic markers				Number of markers recovered[b]			
		LM11 and CML170	LM12 and DMR7	LM13 and CML165	LM14 and CML162	LM11	LM12	LM13	LM14
1	30	10	13	12	8	10 (100)	10 (76.9)	10 (83.3)	8 (100)
2	27	7	12	8	5	7 (100)	11 (92)	7 (87.5)	5 (100)
3	28	12	11	13	10	10 (83.3)	9 (81)	12 (92.3)	10 (100)
4	28	7	8	9	9	6 (85.7)	7 (87.5)	8 (88.8)	7 (77.7)
5	18	10	9	11	13	8 (80)	8 (88.8)	9 (81.8)	11 (84.6)
6	24	11	11	11	14	11 (100)	10 (91)	9 (82)	12 (85.7)
7	36	15	10	9	17	14 (93)	10 (100)	8 (88.8)	15 (88.2)
8	27	7	12	9	9	6 (85.7)	11 (92)	9 (100)	9 (100)
9	29	12	8	13	13	10 (83.3)	8 (100)	13 (100)	10 (76.9)
10	27	8	9	9	9	8 (100)	8 (88.8)	9 (100)	9 (100)
Total	274	103	103	104	107	90 (87.3)	92 (89.3)	94 (90.4)	96 (89.7)

[a] *Chr. no.* = Chromosome number

[b] Values in parenthesis indicates % recurrent parent genome recovery in background selection for each chromosome

Table 2 Segregation pattern of alleles of the *o2* gene in different backcross- and selfed- generations across four crosses

Cross	Generation	Population size	No. of homozygotes for *O2O2*	No. of heterozygotes for *O2o2*	No. of homozygotes for *o2o2*	χ2	*P* value
LM11 × CML170	BC_1F_1	148	81	67	–	1.39^{ns}	0.2384
	BC_2F_1	220	120	100	–	1.83^{ns}	0.1761
	BC_2F_2	150	42	66	42	2.16^{ns}	0.3395
LM12 × DMR7	BC_1F_1	125	69	56	–	1.36^{ns}	0.2435
	BC_2F_1	188	101	87	–	1.04^{ns}	0.3078
	BC_2F_2	200	48	96	56	0.882^{ns}	0.6433
LM13 × CML165	BC_1F_1	210	95	115	–	1.92^{ns}	0.1658
	BC_2F_1	206	92	114	–	2.34^{ns}	0.126
	BC_2F_2	194	54	101	39	2.64^{ns}	0.2671
LM14 × CML162	BC_1F_1	109	63	46	–	2.71^{ns}	0.0997
	BC_2F_1	262	142	120	–	1.85^{ns}	0.1737
	BC_2F_2	160	36	87	37	1.2^{ns}	0.5488

[ns] Non-significant ($p \geq 0.01$), the markers were non-significant and fitted to the normal Mendelian ratio

Phenotypic selection for kernel modification and morphological characteristics

The BC_2F_3 kernels from BC_2F_2 selected plants were segregating for different levels of opaqueness and hardness. In each cross, it was observed that kernel modification could be categorized into five types of kernels with 0%, 25%, 50%, 75% and 100% opaqueness (Fig. S3). The study revealed significant differences within each cross and between the different progenies segregating for endosperm modification indicating that the genetic effects of modifiers vary in different genetic backgrounds and even within the same background contributing adequate variations for these kernels' attributes among the experimental material. The proportion of opaqueness in BC_2F_3 kernels was higher in LM11 and LM14 background, while LM12 and LM13 crosses had a comparatively lesser proportion of opaque seeds (Table S2). BC_2F_3 progenies across crosses were evaluated for various agronomic characters (Table S3). Single plant basis selection was made on different traits

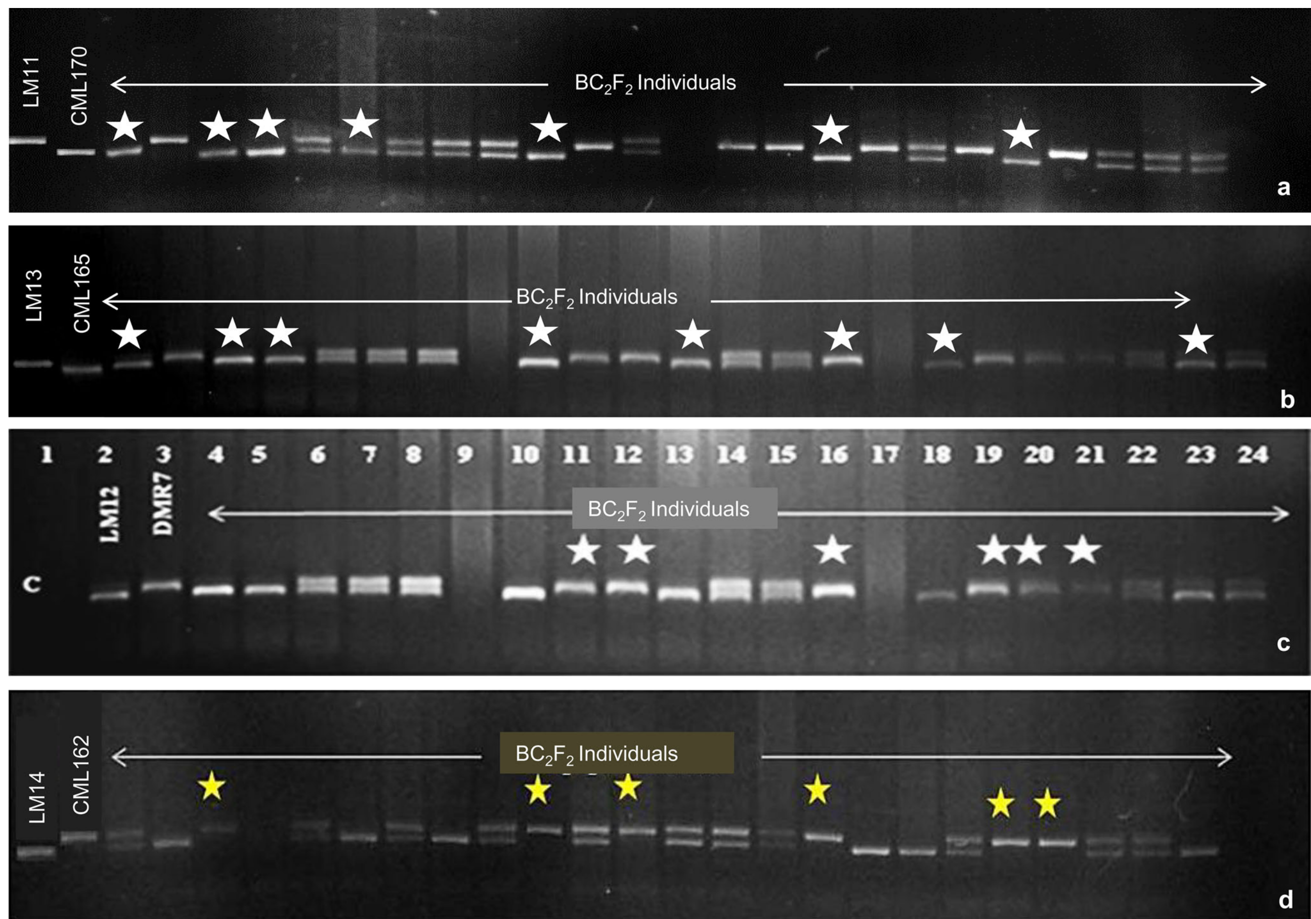

Fig. 2 Foreground selection for identification of homozygous recessive genotypes for *umc1066* locus in LM11 × CML170 **a** and LM13 × CML165 **b**, and for *phi057* locus in LM12 × DMR7 **c** and LM14 × CML162 **d** BC_2F_2 families. The lanes indicated by "star" were homozygotes for *o2* allele

like leaf attitude which was drooping in LM11 and LM12 whereas it was semi-erect in LM13 and LM14; anther glume color was green in three inbreds except LM13 that had pink; anther and silk color was pink in both LM11 and LM13 whereas LM14 had cream anther color and green silk color while LM12 possessed cream anther and silk color. The BC_2F_3 plants having the respective characters of recurrent parent and within range of other traits (plant height, tassel length, ear length) were selected and advanced to BC_2F_4 generation. As it is evident from the data (Table S3) that MAS derived QPM inbreds were close to the original respective inbred line.

Biochemical analysis of BC_2F_4 kernels

Tryptophan content in LM11, LM12, LM13 and LM14 was 0.52, 0.52, 0.48 and 0.53, respectively, whereas, in CML170, DMR7, CML165 and CML162 was 0.97, 1.01, 0.89 and 0.97, respectively. The tryptophan content in BC_2F_4 kernels ranged from 0.76 to 0.95 and 0.72 to 1.00 in LM11/CML170//2*LM11 and LM12/DMR7//2*LM12 crosses, respectively (Fig. 3a–b). For cross LM13/CML165//2*LM13, it ranged from 0.75 to 0.91 (Fig. 3c) whereas it varied from 0.76 to 1.03 in LM14/CML162//2*LM14 cross (Fig. 3d). There was no significant difference with respect to protein content. The protein content varied from 10.88 to 13.24 per cent in BC_2F_4 kernels over all the crosses. LM11, LM12, LM13 and LM14 had protein content of 11.90, 12.30, 10.90 and 12.30 per cent, respectively, whereas DMR7, CML165, CML170 and CML162 had 11.05, 11.03, 12.5 and 11.88 per cent respectively. It was observed that there was no difference with respect to oil and starch content in QPM and non-QPM parents and converted lines. The oil and starch content ranged from 3.9 to 4.5 and 61.2 to 63.5 per cent among the QPM, non-QPM and converted lines.

Reconstitution of QPM hybrids

The selected BC_2F_4 progenies were used in crosses for generation of QPM hybrids.The data on grain yield and quality parameters of the reconstituted hybrids (generated

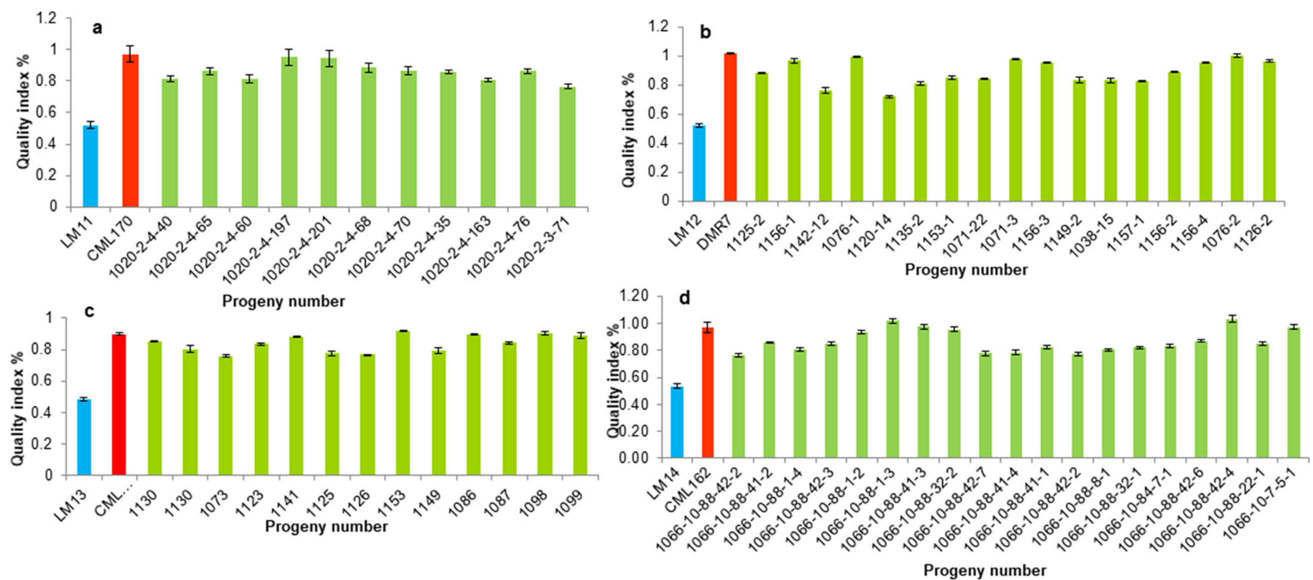

Fig. 3 Levels of tryptophan content in BC_2F_4 kernels of backcross derived QPM lines across four crosses and their parental seeds. The bar indicates the estimation of standard error

by crossing the improved versions of their parental lines) are presented in Table 3. The Buland QPM version viz. JHQPM16; and PMH 1 QPM versions viz. JHQPM 34 and JHQPM 38 were numerically higher yielding than normal respective hybrids but significantly they were on par with each other as well as with hybrid Buland and PMH 1, respectively at both locations. However, Buland QPM versions viz. JHQPM4 and JHQPM20 were significantly at par at Ludhiana location. The QPM versions of hybrids Buland and PMH 1 were significantly higher yielding compared to released QPM checks at both locations. The reconstituted QPM hybrids with the respective original hybrids are shown in Fig. 4. All the reconstituted QPM hybrids had significantly higher tryptophan content ranging from 0.74 to 1.01 compared to 0.51 and 0.48 in Buland and PMH 1original hybrids, respectively.

Discussion

Marker-assisted selection (MAS) has gained significance for improving the effectiveness of plant breeding by precise transfer of gene of interest (foreground selection) and speedy recovery of the recurrent parent genome (background selection) (Babu et al. 2004). Marker assisted foreground selection could be employed successfully for recessive traits that are difficult to detect using conventional breeding approaches (Akhtar et al. 2010). In the present investigation we introgressed *o2* gene in four maize inbreds viz. LM11, LM12, LM13 and LM14 using marker assisted selection and reconstituted QPM hybrid versions of Buland and PMH 1. The foreground markers *phi057* and *umc1066* clearly distinguished non-QPM and QPM lines which were inherited in a codominant manner. Gupta et al. (2013) also validated *o2* specific markers among parents CM212, CM145, CML180 and CML170. This clearly indicates that validation of reported markers for the target trait is mandatory for its' own germplasm to follow further in foreground selection as the allele size and its nature varies with different genetic backgrounds. The observed frequency of 50% individuals with *O2o2* in each backcross generation agreed with the expected frequency of heterozygotes for a single gene in any backcross population. However, for the selection of desirable QPM genotype, we simultaneously performed background selection for recurrent parent genome recovery and phenotypic selection on the selected background recovered heterozygotes. We analyzed 18 to 36 SSR markers per chromosome for parental polymorphism. Here, we report a moderate level of polymorphism between non-QPM and QPM lines. Approximately, 100 polymorphic SSR markers were surveyed in background selection to check the percentage recurrent parent genome recovery in each cross. We covered all bins with equal distribution of markers for all chromosomes, so our results are unbiased with respect to marker data points. The main objective of background selection was at non target loci for recovering maximum proportion of recurrent parent genome with the help of evenly distributed markers on genome in less time (Hospital et al. 1992; Visscher et al. 1996; Frisch et al. 1999a, b). The allocation of individuals with low, moderate, and high recurrent parent genome content in BC_2 population showed unbiased sampling and marker data points.

Table 3 Grain yield and quality traits of reconstituted QPM versions of Buland and PMH 1 hybrid

S. No.	Hybrid	Grain yield (kg/ha)		Tryptophan (%)	Protein (%)
		Ludhiana	Gurdaspur		
Buland QPM versions					
1	JHQPM4	12,066.0	12,169.2	0.99	12.10
2	JHQPM8	11,393.1	11,475.4	0.84	12.00
3	JHQPM10	11,686.9	11,258.9	0.74	11.81
4	JHQPM16	13,762.9	14,822.7	0.97	12.50
5	JHQPM20	12,039.1	12,177.5	0.96	12.42
	Checks				
6	HQPM7	7028.5	–	0.84	10.54
7	HQPM1	7578.0	10,238.7	0.70	11.64
8	Buland	11,505.6	14,676.3	0.51	10.30
	CD (5%)	2270.1	2128.1	3.02	1.83
PMH 1 QPM versions					
1	JHQPM21	6230.5	6422.4	0.90	11.95
2	JHQPM31	6605.6	6755.9	0.93	12.13
3	JHQPM34	7041.1	7976.8	0.95	11.85
4	JHQPM35	6367.9	6466.3	1.01	11.69
5	JHQPM38	6863.7	7095.8	0.94	11.77
	Checks				
6	HQPM 1	4542.5	5773.9	0.74	9.92
7	HQPM7	4653.8	–	0.9	9.87
8	PMH1	6511.5	6402.9	0.48	10.11
	CD (5%)	1356.3	3950.8	0.02	1.70

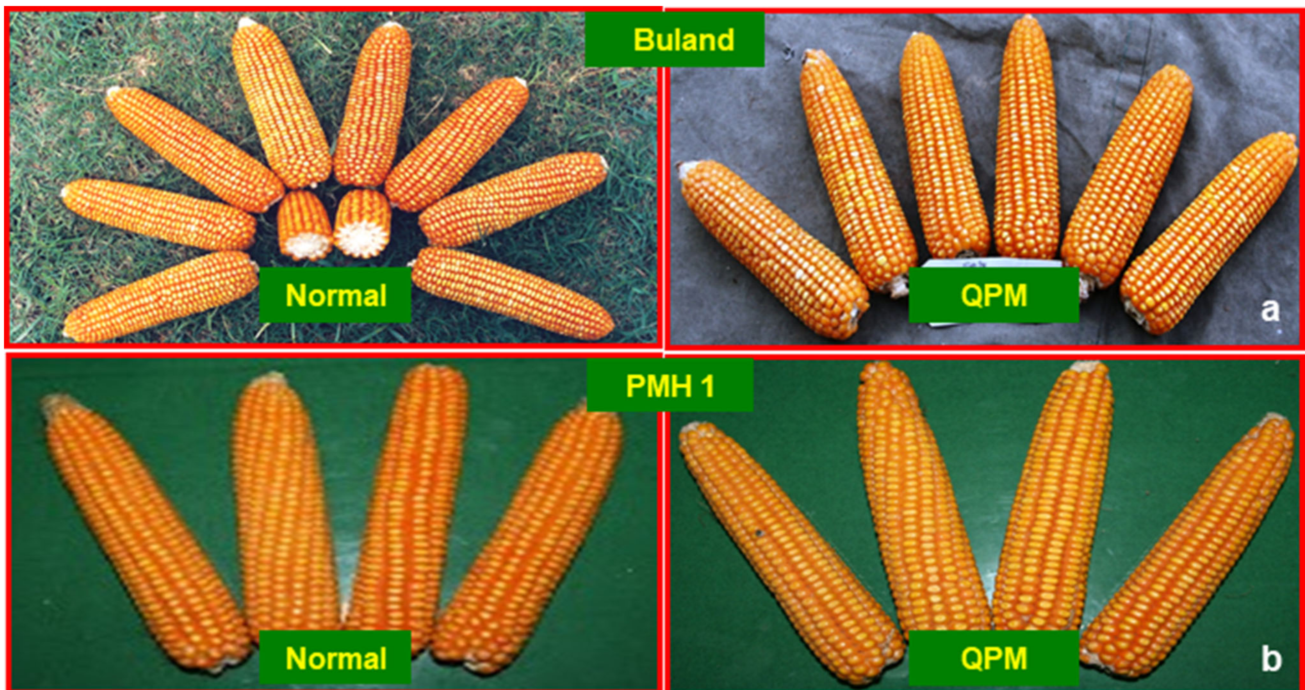

Fig. 4 Comparison of cob characteristics of normal and QPM version hybrids. **a** Cobs of Buland, normal versus QPM. **b** Cobs of PMH 1, normal versus QPM

The challenge in QPM breeding is restoring kernel vitreousness because of the complexity of modifiers. Endosperm modification was quantitatively inherited (Bajarnason and Vasal 1992; Lopes and Larkin 1995), while the degree of kernel vitreousness and increased synthesis of γ-zein in modified endosperms were both dosage-dependent and directly correlated (Lopes and Larkin 1991). Several reports on kernel modification indicated the preponderance of additive gene action (Vasal et al. 1980, 1993; Hohlset al. 1996; Bhatnagar et al. 2004). QTLs for endosperm modifiers have been mapped on chromosomes 1, 5, 7, 9 and 10 (Babu et al. 2015; Holding et al. 2008, 2011). One major QTL present on chromosome 7 affects gene expression and protein accumulation of 27-kD γ-zein. The elevated accumulation of the 27-kDa γ-zein in QPM endosperm is associated with an increase in the number and size of protein bodies, and its interaction with amylopectin molecules at the surface links starch granules results in formation of vitreous endosperm in the *o2* mutant background (Wu et al. 2010). The 27-kD γ-zein protein increase is conferred by a duplication event at 27-kD γ-zein locus which is common across all QPMs but other minor effect QTLs are not common across all QPMs (Liu et al. 2016). As a result, the beneficial alleles of the γ-zein production strongly rely on phenotypic selection using a rapid, low-cost, light table method. In the present study, the degree of opaqueness varied with each cross due to the diffraction of light caused by the air spaces left during packaging of protein and starch granules in the endosperm (Wu and Messing 2010). The study revealed significant differences within and between the converted QPM lines for endosperm modification that could be attributed to different types of kernel modifiers present in different genetic backgrounds. It was observed that opacity of kernels of the improved versions of LM11 were higher followed by QPM versions of LM14 (Table S2). It means that non-QPM inbreds, LM12 and LM13 contributed more kernel modifier genes than LM11 and LM14. Moreover, the donor parent in each cross was contributing to kernel viterousness. Thus, there might be several minor genes for kernel modification, and these are inherited not only from donors but also from recurrent parents (Gupta et al. 2013; Pandey et al. 2015). Therefore, it is necessary to test different cross combinations to combine the two parents of a hybrid with different kernel modifier genes. This result in genetic complementation of kernel modifier genes in the QPM cross combinations.

The genetic enhancement of lysine and tryptophan in the converted QPM lines was accomplished by the presence of *o2* allele as well as amino acid modifiers. At least three genes associated with lysine level have been mapped to locations on chromosomes 2, 4, and 7 (Wang et al. 2001). The QTL on chromosome 2L is coincident with genes encoding two aspartate kinase (*ASK*) enzymes, which control important steps in amino acid biosynthesis and lysine degradation pathway. Ten significant and one suggestive QTL for free amino acid (FAA) content were identified on all 10 chromosomes (Wu et al. 2002). Six QTLs associated with tryptophan content were identified on chromosomes 5, 7 and 9 (Babu et al. 2015). One QTL was detected near *ASK3* gene on chromosome 5 and other near *Wx1* locus on chromosome 9 (Babu et al. 2015). A negative correlation was observed between endosperm texture trait and amino acid content (Gutierrez-Rojas et al. 2008). Therefore, their regular monitoring at each step is essential as one could end up with a maize cultivar having the *o2o2* genotype with lysine and tryptophan levels equivalent to those in normal maize (Krivanek et al. 2007). Estimation of tryptophan was used as a single parameter for evaluating the nutritional quality of the protein (Hernandez and Bates 1969; Villegas et al. 1992) as relationship between lysine and tryptophan are highly correlated and normally the value of the lysine is four times that of tryptophan (Moro et al. 1996). It has been suggested that protein, tryptophan, and quality index must be above the acceptance limits when inferring the results for selection of QPM (Vivek et al. 2008). In all the analyzed samples, protein content was within limits for all QPM and non-QPM genotypes. Significant differences in tryptophan content between normal and *o2* lines were observed as expected. The enhancement in tryptophan was 43% to 94% in the selected progenies over their respective recurrent parents. The tryptophan content varied among improved inbred lines and donors despite the presence of the same *o2* allele. The variation for tryptophan content in the progenies was due to the presence of different types of amino acid modifiers. The tryptophan content of some progenies was at par to donors which would be due to presence of the right dosage and blend of amino acid modifiers from both parents. Similar results were obtained by previous studies (Gupta et al. 2013; Liu et al. 2015). It could be concluded that a breeder must bring favorable amino acid modifiers along-with introgression of *o2* for improving the tryptophan content of maize inbred lines.

Phenotypic selection was practiced in the marker selected *o2* individuals in the BC_1F_1, BC_2F_1 and BC_2F_3 progenies. The MAS technology along-with judicious phenotypic selection in each generation resulted in combining the desirable agronomic traits with enriched protein quality as well as hard endosperm trait. The stable expressions of these agronomic traits have been confirmed in BC_2F_4 progenies and further selection was done on the single plant basis for advancement of generation to BC_2F_5. Phenotypic evaluation in addition to the background selection is also useful to find the recovery percentage of recurrent parents (Manna et al. 2005; Choudhary 2014;

Hossain et al. 2018). Phenotypic selection among the improved lines presented more than 90% of recovery of the recurrent parents with respect to morphological traits. We recovered inbred lines for *o2* allele with 87–90 percent recurrent parent genome recovery coupled with higher level of tryptophan content.

The converted QPM version of LM11, LM12, LM13 and LM14 were involved in generation of QPM versions of Buland and PMH 1 hybrids. The evaluation of QPM hybrids showed that some of the versions were at par with checks and original hybrids both in yield as well as tryptophan content. The improved QPM maize hybrids viz. Buland and PMH 1 reconstituted by our group showed an average 76% and 97% increase in tryptophan content over the original hybrids, respectively. In conclusion, QPM hybrids could possibly be grown in India without any difference in grain yield and to greater gain in terms of grain nutrition compared with normal maize as the biological value of QPM protein is usually almost double than normal maize protein (Bressani 1990; Graham et al. 1980).

The combined strategy of foreground, background and phenotypic selection has resulted in the successful conversion of QPM genotypes in cost effective manner. The promising QPM versions are currently being used in the maize biofortification programme to further enrich the nutritional quality of maize. The most important goal of QPM research has been to reduce malnutrition through direct human consumption. It is expected that the greater impact will accrue out of development and dissemination of improved QPM hybrids and may drive significant nutritional and economic benefits for the society.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Akhtar S, Bhat MA, Wani SA, Bhat KA, Chalkoo S, Miir MS, Wani SA (2010) Marker assisted selection in rice. J Phytol 10:66–81

Azevedo RA, Arruda P (2010) High-lysine maize: the key discoveries that have made it possible. Amino Acids 39:979–989

Babu BK, Agrawal P, Saha S, Gupta H (2015) Mapping QTLs for *opaque2* modifiers influencing the tryptophan content in quality protein maize using genomic and candidate gene-based SSRs of lysine and tryptophan metabolic pathway. Plant Cell Rep 34:37–45

Babu R, Nair SK, Kumar A, Venkatesh S, Sekhar JC, Singh NN, Srinivasan G, Gupta HS (2005) Two generation marker-aided backcrossing for rapid conversion of normal maize lines to quality protein maize (QPM). Theor Appl Genet 111:888–897

Babu R, Nair SK, Prasanna BM, Gupta HS (2004) Integrating marker assisted selection in crop breeding-prospects and challenges. Curr Sci 87:607–619

Bajarnason M, Vasal SK (1992) Breeding of quality protein maize (QPM). Plant Breed 9:181–216

Bhatnagar S, Betran FJ, Rooney LW (2004) Combining abilities of quality protein maize inbreds. Crop Sci 44:1997–2005

Bressani R (1990) Quality protein maize. American Association of Cereal Chemists, Saint Paul, Minnesota, United States, pp 148–150

Choudhary M (2014) Morphological and biochemical characterisation of *crtRB1* introgressed MAS derived β-carotene rich inbreds in maize (*Zea mays* L.) Dissertation, IARI India

Damerval C, Vienne D (1993) Quantification of dominance for proteins pleiotropically affected by *opaque-2* in maize. Heredity 70:38–51

Frisch M, Bohn M, Melchinger AE (1999a) Comparison of selection strategies for marker assisted backcrossing of a gene. Crop Sci 39:1295–1301

Frisch M, Bohn M, Melchinger AE (1999b) Minimum sample size and optimal positioning of flanking markers in marker assisted backcrossing for transfer of a target gene. Crop Sci 39:967–975

Gaziola SA, Alessi ES, Guimaraes PEO, Damerval C, Azevedo RA (1999) Quality protein maize: a biochemical study of enzymes involved in metabolism. J Agri Food Chem 47:1268–1275

Geevers HO, Lake JK (1992) Development of modified *opaque2* maize in South Africa. In: Mertz ET (ed) Quality Protein Maize. American Association of Cereal Chemists, Saint Paul, Minnesota, United States, pp 49–78

Graham GG, Placko RP, Maclean WC (1980) Nutritional value of normal, *opaque-2* and *sugary-2opaque-2* maize hybrids for children and infants 2 Plasma free amino acids. J Nutr 110:1070–1074

Gupta HS, Raman B, Agrawal PK, Mahajan V, Hossain F, Thirunavukkarasu N (2013) Accelerated development of quality protein maize hybrid through marker assisted introgression of *opaque-2* allele. Plant Breed 132:77–82

Gutierrez-Rojas A, Scott MP, Leyva OR, Menz M, Betran J (2008) Phenotypic characterization of quality protein maize endosperm modification and amino acid contents in a segregating recombinant inbred population. Crop Sci 48:1714–1722

Habben JE, Kirlies AW, Larkins BA (1993) The origin of lysine containing proteins in *opaque2* maize endosperm. Plant Mol Biol 23:825–838

Hernandez HH, Bates LS (1969) A modified method for a rapid tryptophan analysis in maize. Research Bulletin, CIMMYT, Mexico, pp 1–7

Hohls T, Shanahan PE, Clarke GP, Gevers HO (1996) Genetic control of kernel modification found in South African quality protein maize inbred lines. Euphytica 87:103–109

Holding DR, Hunter BG, Chung T, Gibbon BC, Ford CF, Bharti AK, Messing J, Hamaker BR, Larkins BA (2008) Genetic analysis of *opaque-2* modifier loci in quality protein maize. Theor App Genet 117:157–170

Holding DR, Hunter BG, Klingler JP, Wu S, Guo X, Gibbon BC, Wu R, Schulze JM, Jung R, Larkins BA (2011) Characterization of *opaque2* modifier QTLs and candidate genes in recombinant inbred lines derived from the K0326Y quality protein maize inbred. Theor Appl Genet 122:783–794

Hospital F, Chevalet C, Mulsant P (1992) Using markers in gene introgression breeding programs. Genetics 132:199–210

Hossain F, Muthusamy V, Pandey N (2018) Marker-assisted introgression of *opaque2* allele for rapid conversion of elite hybrids into quality protein maize. J Genet 97:287–298

Kemper EL, Neto GC, Papes F, Moraes KC, Leite A, Arruda P (1999) The role of *opaque2* in the control of lysine degrading activities in developing maize endosperm. Plant Cell 11:1981–1994

Krivanek AF, De Grote H, Gunaratna NS, Diallo AO, Friesen D (2007) Breeding and disseminating quality protein maize (QPM) for Africa. Afr J Biotechnol 6:312–324

Lauderdale J (2000) Issues regarding targeting and adoption of quality protein maize (QPM) in CIMMYT Economics Working Paper 00–02. CIMMYT, Mexico

Li C, Qiao Z, Qi W, Wang Q, Yuan Y, Yang X, Tang Y, Mei B, Lv Y, Zhao H, Xiao H, Song R (2015) Genome-wide characterization of cis-acting DNA targets reveals the transcriptional regulatory framework of *opaque2* in maize. Plant Cell 27:532–545

Liu H, Shi J, Sun C, Gong H, Fan X, Qiu F et al (2016) Gene duplication confers enhanced expression of 27-kDa γ-zein for endosperm modification in quality protein maize. Proc Natl Acad Sci USA 113:4964–4969

Liu L, Jeffers D, Zhang Y, Ding M, Chen W, Kang MS (2015) Introgression of the *crtRB1* gene into quality protein maize inbred lines using molecular markers. Mol Breed 35:154–165

Lopes MA, Larkin BA (1991) Gamma-Zein content is related to endosperm modification in Quality Protein Maize. Crop Sci 31:1655–1662

Lopes MA, Larkin BA (1995) Genetic analysis of *opaque2* modifier gene activity in maize endosperm. Theor Appl Genet 119:274–281

Manna R, Okello OK, Imanywoha J, Pixley K, Edema R (2005) Enhancing introgression of the *opaque-2* trait into elite maize lines using simple sequence repeats. Afr Crop Sci J 13:215–226

McWhirter KS (1971) A floury endosperm, high lysine locus on chromosome 10. Maize Genetic Coop Newslett 45:184

Mertz ET, Bates LS, Nelson OE (1964) Mutant gene that changes protein composition and increases lysine content of maize endosperm. Science 145:279–280

Moro GL, Habben JE, Hamaker BR, Larkins BA (1996) Characterization of the variability in lysine content for normal and *opaque2* maize endosperm. Crop Sci 36:1651–1659

Nelson OE (1969) Genetic modification of protein quality in plants. Adv Agron 21:171–194

Nelson OE (1979) More precise linkage data on *fl3*. Maize Genetic Coop Newslett 53:56

Nelson OE (1981) The mutations *opaque-9* through *opaque-13*. Maize Genetic Coop, Newslett 55:68

Nelson OE, Mertz ET, Bates LS (1965) Second mutant gene affecting the amino acid pattern of maize endosperm proteins. Science 150:1469–1470

Ortega EI, Bates LS (1983) Biochemical and agronomic studies of two modified hard-endosperm *opaque2* mutants. Cereal Chem 60:107–126

Pandey N, Hossain F, Kumar K, Vishwakarma AK, Muthusamy V, Saha S (2015) Molecular characterization of endosperm—and amino acids—modifications among quality protein maize inbreds. Plant Breed 135:47–54

Prasanna BM, Vasal SK, Kassahun B, Singh NN (2001) Quality protein maize. Curr Sci 81:1308–1319

Rossi V, Motto M, Pellegrini L (1997) Analysis of the methylation pattern of the maize *opaque-2* (*O2*) promoter and in vitro binding studies indicate that the *O2* B-Zip protein and other endosperm factors can bind to methylated target sequences. J Biol Chem 272:13758–13765

Saghai-Maroof HA, Soliman KM, Jorgensen AR, Allard RW (1984) Ribosomal DNA spacer length polymorphism in barley: mendelian inheritance, chromosomal location and population dynamics. Proc Natl Acad Sci USA 81:8014–8018

Schmidt RJ, Burr FA, Aukerman MJ, Burr B (1990) Maize regulatory gene *opaque2* encodes protein with a "leucine zipper" motif that binds to zein DNA. Proc Natl Acad Sci USA 87:46–50

Vasal SK (2000) The quality protein maize story. Food Nutr Bull 21(4):445–450

Vasal SK (2002) Quality protein maize: overcoming the hurdles. J Crop Prod 6:193–227

Vasal SK, Srinivasan G, Pandey S, Gonzalez CF, Crossa J (1993) Heterosis and combining ability of CIMMYT's quality protein maize germplasm: I. Low Land tropical Crop Sci 33:46–51

Vasal SK, Villegas E, Bjarnason M, Gelow B, Goertz P (1980) Genetic modifiers and breeding strategies in developing hard endosperm opaque material. In: Pollmer WG, Phillips RH (eds) Improvement of quality traits of maize for grain and silage used. Publishers Mortinus Nijhoff, London, pp 37–73

Vikal Y, Chawla JS (2014) Molecular interventions for enhancing the protein quality of maize. In: Chaudhary D et al (eds) Maize: nutrition dynamics and novel uses. Springer, Berlin, pp 49–61

Vikal Y, Kaur G, Kaur R, Kaur D, Gill GK, Chawla JS (2013) Molecular characterization of quality protein maize inbred lines using SSR markers. Crop Improv 40:129–139

Villegas E, Mertz ET (1971) Chemical screening methods for maize protein quality at CIMMYT. CIMMYT, Mexico, pp 1–14

Villegas E, Vasal SK, Bjarnarson M (1992) Quality protein maize: what it is and how was it developed. In: Mertz ET (ed) Quality Protein Maize. American Association of Cereal Chemists, Saint Paul, Minnesota, United States, pp 27–48

Visscher PM, Haley CS, Thompson R (1996) Marker assisted introgression in backcross breeding programs. Genetics 144:1923–1932

Vivek BS, Krivanek AF, Rojas PN, Afriyie TS, Diallo AO (2008) Breeding quality protein maize (QPM): protocols for developing QPM cultivars. CIMMYT, Mexico

Wallace JC, Lopes MA, Paiva E, Larkins BA (1990) New method for extraction and quantitation of zeins reveal a high content of gamma-zein in modified *opaque2* maize. Plant Physiol 92:191–196

Wang X, Stumpf DK, Lark ins BA (2001) Aspartate kinase 2. A candidate gene of a quantitative trait locus influencing free amino acid content in maize endosperm. Plant Physiol 125:1778–1787

Wu RL, Lou XY, Ma CX, Wang XL, Larkins BA, Casella G (2002) An improved genetic model generates high resolution mapping of QTL for protein quality in maize endosperm. Proc Natl Acad Sci USA 99:11281–11286

Wu Y, Holding DR, Messing J (2010) γ-zeins are essential for endosperm modification in quality protein maize. Proc Natl Acad Sci USA 107:12810–12815

Wu Y, Messing J (2010) RNA interference-mediated change in protein body morphology and seed opacity through loss of different zein proteins. Plant Physiol 153:337–347

Yang W, Zheng Y, Ni S, Wu J (2004) Recessive allelic variations of three microsatellite sites within the *O*2 gene in maize. Plant Mol Bio Rep 22:361–374

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