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RESEARCH ARTICLE

Marker-assisted introgression of *opaque2* allele for rapid conversion of elite hybrids into quality protein maize

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Abstract. Maize is a valuable source of food and feed worldwide. Maize endosperm protein is, however nutritionally poor due to the reduced levels of two essential amino acids, lysine and tryptophan. In this study, recessive *opaque2* (*o*2) allele that confers enhanced endosperm lysine and tryptophan, was introgressed using marker-assisted backcross breeding into three normal inbred lines (*HKI323, HKI1105* and *HKI1128*). These are the parental lines of three popular medium-maturing single cross hybrids (*HM4, HM8* and *HM9*) in India. Gene-based simple sequence repeat (SSR) markers (*umc1066* and *phi057*) were successfully deployed for introgression of *o*2 allele. Background selection using genome-based SSRs helped in recovering > 96% of recurrent parent genome. The newly developed quality protein maize (QPM) inbreds showed modified kernels (25–50% opaqueness) coupled with high degree of phenotypic resemblance to the respective recipient lines, including grain yield. In addition, endosperm protein quality showed increased lysine and tryptophan in the inbreds to the range of 52–95% and 47–118%, respectively. The reconstituted QPM hybrids recorded significant enhancement of endosperm lysine (48–74%) and tryptophan (55–100%) in the endosperm. The QPM hybrids exhibited high phenotypic similarity with the original hybrids for morphological and yield contributing traits along with responses to some major diseases like turcicum leaf blight and maydis leaf blight. The grain yield of QPM hybrids was at par with their original versions under multilocation testing. These elite, high-yielding QPM hybrids with improved protein quality have been released and notified for commercial cultivation, and hold significant promise for improving nutritional security.

Keywords. biofortification; essential amino acids; *opaque2*; quality protein maize; marker-assisted selection.

Introduction

Protein-energy malnutrition (PEM) has emerged as a major health problem, especially in the developing world (Temba *et al.* 2016). Maize provides a significant amount of total calorie to the human populations worldwide. Energy requirement to the tune of 62% in Mesoamerica, 43% in eastern and southern Africa, 22% in west and central Africa, and 28% in the Andean region, comes from maize (Shiferaw *et al.* 2011). It is also a preferred choice as a food in many of the tribal belts, especially in the north eastern states. A major portion (60–70%) of maize grains produced worldwide is used for animal consumption. Maize thus serves as an important source of plant protein and total calorie both directly and indirectly. Maize endosperm protein is, however, known to be poor in nutritional value due to low amount of essential amino acids, lysine (2.0% in protein) and tryptophan (0.4% in protein), and their concentration is nearly half of the level recommended for human nutrition (Mertz *et al.* 1964; Prasanna *et al.* 2001). Since, humans and monogastric animals like poultry cannot synthesize these amino acids in their body, healthy diets therefore must include the alternate sources of lysine and tryptophan (Bjarnason and Vasal 1992; Gupta *et al.* 2013). Among various strategies, biofortification

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turns out to be the most sustainable and cost-effective solution to provide micronutrients in natural form (Bouis *et al.* 2011; Gupta *et al.* 2015).

The recessive *opaque2* (*o*2) mutant has been successfully utilized in the breeding programme for enhancement of protein quality (Vasal *et al.* 1980). Initially, maize cultivars with *o*2 mutation was not preferred by farmers and consumers, due to soft and opaque endosperm, increased susceptibility to insect-pest and diseases, and breakage of grains during mechanical processing (Bjarnason and Vasal 1992). Later, endosperm modifier genes that confer hard endosperm in the *o*2 background were introgressed at CIMMYT, Mexico (Villegas *et al.* 1992) and University of Natal, South Africa (Geevers and Lake 1992). This eventually led to the development of nutritionally enriched hard endosperm maize, popularly phrased as 'quality protein maize (QPM)' (Vasal *et al.* 1980).

Globally, a large number of normal maize hybrids have been released and commercialized. In contrast, the germplasm base of QPM is quite narrow, and substantially lesser number of genetically diverse QPM hybrids are available. In India, nearly a dozen QPM hybrids has been released, compared to more than hundred non-QPM / normal maize hybrids (Yadav *et al.* 2015). It is therefore necessary to develop diverse QPM varieties across different maturity groups and agroecology. The conventional approach takes 10–15 years or more to implement this programme. Conversion of elite normal maize hybrids into QPM requires significantly lesser time, primarily due to tested combining ability, heterosis and adaptability of the already released hybrid (Prasanna *et al.* 2010). Introgression of a recessive allele through conventional backcross breeding involves 6–7 generations of backcrossing. This time can be significantly reduced to two backcrosses by molecular marker-assisted backcross breeding (MABB) (Babu *et al.* 2005; Muthusamy *et al.* 2014). Here, we report rapid conversion of three popular medium-maturity single-cross hybrids released in India, *HM4*, *HM8* and *HM9*, to QPM using MABB.

Materials and methods

Plant materials

The genetic materials comprised of three elite normal/non QPM maize inbreds, *HKI323, HKI1105* and *HKI1128* having low level of lysine (1.76–2.08% in protein) and tryptophan (0.35–0.52% in protein) (table 1). These are the parents of three commercial medium maturing single cross maize hybrids in India (*HM4* (*HKI1105*×*HKI323*), *HM8* (*HKI1105*×*HKI161*) and *HM9* (*HKI1105*×*HKI1128*)). *HKI161* possesses *o*2 allele and is a QPM inbred. *HM4* is adapted to north western plain zone (NWPZ), while *HM8* and *HM9* are for peninsular zone (PZ) and north eastern plain zone (NEPZ) of India (Kaul *et al.* 2009).

Table 1. Details of the recurrent and donor parents used in the study.

Name*	Derived from
Recurrent parents	
HKI323	CIMMYT Pool 28
HKI1105	*Cargil 633*
HKI1128	Hybrid from a farmer's field
Donor parents	
HKI161	*CML161*
CML161	*G25QC18H520*
HKI193-1	*CML193*

*The source of all is CCSHAU, except for CML161 which was obtained from CIMMYT, Mexico.

CML161 (CIMMYT QPM inbred), *HKI161* and *HKI193-1* (QPM inbreds selected from *CML161* and *CML193*, respectively at Uchani Centre, Chaudhary Charan Singh Haryana Agricultural University (CCSHAU), India) with high levels of endosperm lysine (3.32–3.80% in protein) and tryptophan (0.74–0.85% in protein), and desirable endosperm modification served as the donors for introgression of *o*2 allele.

Target allele for introgression

Two SSRs, *umc1066* and *phi057* are present in first exon and sixth exon, respectively, of *O*2 gene present on chromosome 7 (Yang *et al.* 2004). The primer sequences are: *umc1066* = forward (F): 5′-ATGGAGCACGTCATCTCA ATGG-3′; reverse (R): 5′-AGCAGCAGCAACGTCTAT GACACT-3′ and *phi057* = F: 5′-CTCATCAGTGCCGTC GTCCAT-3′ and R: 5′-CAGTCGCAAGAAACCGTTGC C-3′. Polymorphic SSRs between respective recurrent and donor parents were used for marker-assisted selection (MAS) of the *o*2 allele.

DNA isolation and polymerase chain reaction (PCR)

Genomic DNA was isolated from young seedlings using the standard CTAB procedure (Murray and Thompson 1980). PCR reaction (Bio-Rad, California, USA) was carried out in 10 μL of a reaction mixture containing 2 μL of 20 ng/μL genomic DNA as the template, 2 mM $MgCl_2$, 1 mM dNTPs, 2 μM of the primer pair (F and R), and 1.5 U *Taq* polymerase (GeNei, Mumbai, India). Touch down procedure standardized at Maize Genetics Unit, ICAR-Indian Agricultural Research Institute (IARI), New Delhi, was used for PCR amplification (Pandey *et al.* 2015). The resulting PCR amplicons were resolved in 4% agarose gel for 4 h. The resolved amplified products were visualized using a gel documentation system (AlphaInnotech, California, USA).

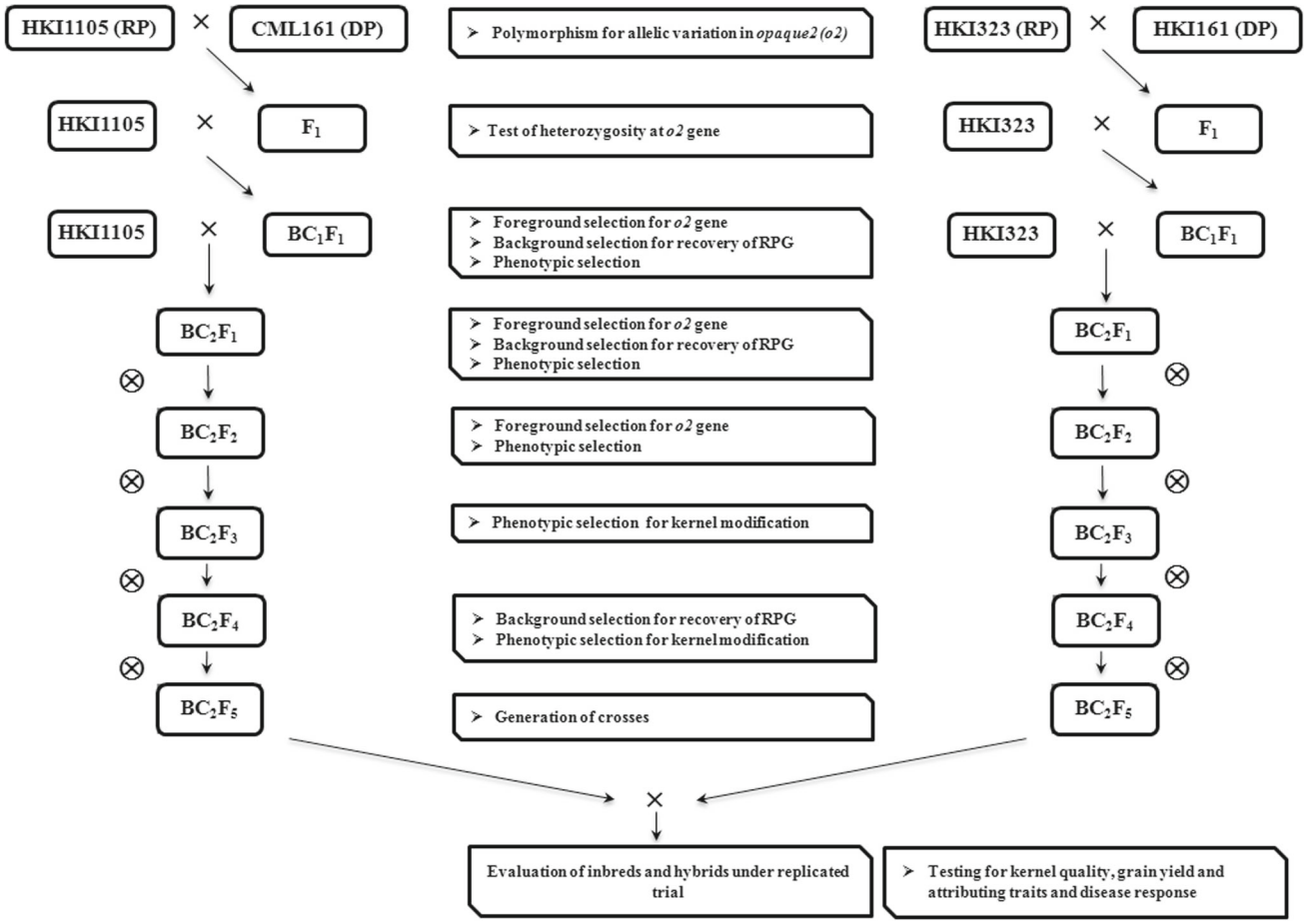

Figure 1. Marker-assisted backcross breeding scheme adopted for conversion of normal maize hybrids into QPM versions.

MABB strategy

For MABB (figure 1), recurrent parents (as females) and donors (as males) were crossed in 2009 rainy season (July to October) at IARI experimental farm, New Delhi. The F_1s were raised at the Maize Winter Nursery Centre (off season nursery), Hyderabad, India, during 2009–2010 winter season (December to April). Heterozygosity of the F_1s was tested using the *o*2-specific marker, and the true F_1s were backcrossed as male parents to their respective recurrent parents. BC_1F_1 progenies were grown at Delhi during the rainy season in 2010, and foreground selection was carried out using the *o*2-gene-specific marker(s). Desirable plants with high recovery of the recurrent parent genome (RPG) and morphological similarity to recurrent parents were further backcrossed to raise the BC_2F_1 population at Hyderabad during 2010–2011 winter season. The selected plants with high RPG and phenotypic similarity to their recurrent parents were selfed. BC_2F_2 populations were raised at Delhi during 2011 rainy season, and selected plants were selfed to advance progenies. Backcross progenies of *HKI323*×*HKI161* and *HKI1105*×*CML161* were generated as per the above procedure. However, for *HKI1128*×*HKI193-1*, BC_1F_1 seeds could not be generated during winter of 2009–2010 owing to the nonsynchrony of flowering; hence, fresh crosses were generated and F_1s were planted at Delhi during 2010 rainy season, and all backcross progenies were eventually raised one generation later compared to other two inbreds.

Marker-assisted foreground selection

Foreground selection was employed in BC_1F_1, BC_2F_1 and BC_2F_2 generations using the marker specific to *o*2 allele. SSRs, *umc1066* and *phi057* were used for selection of the foreground positive plants. Heterozygous plants (*O2*/*o2*) were selected in the BC_1F_1 and BC_2F_1, and homozygotes (*o2*/*o2*) were selected in BC_2F_2. Chi-square (χ^2) test was performed to test the goodness of fit of the observed segregation pattern at the *o*2 locus in each of the generations.

Marker-assisted background selection

A set of > 350 genomewide SSRs distributed throughout the maize genome was used for identifying polymorphic

markers between the respective recurrent and donor parents. The sequences of the SSR primers were adapted from the maize genome database (www.maizegdb.org) and custom synthesized (Sigma Tech., USA). These polymorphic SSR markers were employed in each of the BC_1F_1 and BC_2F_1 generations of the three crosses to recover the RPG. The final recovery of RPG, across genetic backgrounds, was determined in the BC_2F_4 generation.

Endosperm modification

Selfed seeds (BC_2F_3 onwards) from homozygous plants (*o*2/*o*2) were selected and analysed for the degree of opaqueness using a standard light box (Vasal *et al.* 1980). For analysis of endosperm modification, the back-lit kernels were rated on a scale of 0 to 100, with each number indicating per cent opaqueness. For instance, '100' stands for 100% opaque while '0' for 100% vitreous/hard (Hossain *et al.* 2008). Grains with minimal degree of opaqueness were selected and used for advancement of homozygous progenies (*o*2/*o*2).

Phenotypic characterization of MAS-derived inbreds

The MAS-derived inbreds along with the original inbreds were evaluated at Delhi, during 2013 and 2014 rainy season in two replications each having two-rows/entry. Inbreds were characterized for 31 morphological characters (PPVFRA 2007) and 12 grain yield-related traits. Plants were raised in 3 m length row with a plant to plant distance of 20 cm, and row to row distance of 75 cm. Standard agronomic practices like application of 10–15 t of farmyard manure, 150: 80: 60 kg of N: P: K, and 20–25 kg $ZnSO_4$ per ha in soil, and 6–8 irrigations depending upon the requirement were given, to raise the crop.

Evaluation of MAS-derived hybrids

Since the hybrids targeted for improvement are adaptable for cultivation in rainy season, crosses were generated in winter at off season nursery, and reconstituted hybrids were evaluated in the following rainy season. In rainy season, seeds of selected MAS-derived QPM inbreds were increased, and progenies (BC_2F_5 for *HKI323* and *HKI1105*, and BC_2F_4 for *HKI1128*) were crossed to reconstitute the QPM version of original hybrid at Hyderabad, during 2012–2013 winter season. *HKI161* being a QPM inbred, was directly used as a parent for generating QPM version of *HM8*. The crosses along with original hybrids were evaluated in two replications, each having two-rows/entry at Delhi during 2013 rainy season. Standard agronomic practices used for raising inbreds were also followed to raise the hybrids. The hybrids were characterized for 31 morphological characters (PPVFRA 2007) and 12 grain yield-related traits.

Estimation of lysine and tryptophan in endosperm protein

Inbred trials conducted for assessing the grain yield and component traits at Delhi during 2013 and 2014 were used for estimation of lysine and tryptophan in endosperm protein. For hybrids, the yield trial conducted in Delhi during 2013 was used for quality analyses. In addition, a separate hybrid trial was constituted at Delhi in 2014 only for quality analyses. Two to three plants in each entry of the trials were self-pollinated, and selfed grains were used for estimation of grain quality. Total endosperm protein was estimated using the micro-Kjeldahl procedure (AOAC 1965). The concentration of lysine and tryptophan in endosperm flour was measured using UPLC (Dionex Ultimate 3000, Thermo Scientific) at Maize Genetics Unit, IARI, New Delhi (Sarika *et al.* 2016). Degermed endosperm flour per sample with three replications was used. Acid hydrolysis was done for lysine, while for tryptophan alkaline hydrolysis was performed. Samples were eluted through Acclaim 120 C_{18} column (5 μm, 120 Å, 4.6 $\times$ 150 mm, Thermo Scientific) and detected with RS photodiode array detector (PDA) with absorbance at 265 and 280 nm wavelength respectively. Concentration of amino acids in each sample was estimated by standard regression using external standards (AAS-18, Sigma Aldrich, USA).

Evaluation of MAS-derived hybrids in multilocation-based national trials

The MAS-derived experimental crosses evaluated at Delhi during 2013 rainy season were further nominated as 'essentially derived variety' (EDV) and tested under the AICRP-Maize, coordinated by the ICAR-Indian Institute of Maize Research (IIMR), New Delhi. Under this system, each entry was coded and the trial for each of the hybrids was undertaken at 3–12 designated locations of their respective zone of adaptation. The entries were evaluated in complete randomized block design (three replications and having four rows/entry/replications) for the two consecutive years (2014 and 2015 rainy seasons) (Annual Progress Report, Kharif Maize 2015, 2016). Various agronomic traits including grain yield were recorded. Hybrids were also evaluated for their responses to diseases like, maydis leaf blight, turcicum leaf blight, banded leaf and sheath blight, polysora rust, common rust, charcoal rot, fusarium stalk rot, sorghum downy mildew, Rajasthan downy mildew and bacterial stalk rot.

Results

Marker polymorphism

The *umc1066* was polymorphic between recurrent parents (*HKI323* and *HKI1128*) and donor QPM inbreds (*HKI161* and *HKI193-1*), respectively. For *HKI1105* and *CML161*,

phi057 was used as a polymorphic marker. Genome-based SSR markers, 351–463, distributed among 10 chromosomes were screened between the recipient and donor parents and 30–42% was polymorphic across three crosses (table 2).

Marker-assisted introgression of o2 allele

***BC_1F_1 generation*:** Foreground selection in BC_1F_1 resulted in the identification of 21 heterozygous plants in *HKI323*×*HKI161*, 32 in *HKI1105*×*CML161* and 50 in *HKI1128*×*HKI193-1* populations (table 3). Segregation of *o*2 allele in all the three populations deviated from the expected Mendelian ratio of 1:1 (table 3), while recovery of RPG varied from 63.26 to 91.9% (table 4). Two plants each in *HKI323*-based (73.6 and 74.3% RPG), *HKI1105*-based (76.3 and 77.0% RPG) and *HKI1128*-based (85.5 and 90.0% RPG) populations were selected for further advancement (table 4).

***BC_2F_1 generation*:** A total of 53 heterozygous plants (*O*2/*o*2) in *HKI323*×*HKI161*, while 57 in *HKI1105*×*CML161* and 68 in *HKI1128*×*HKI193-1* were identified (table 3). Significant segregation distortion of *o*2 allele was observed in the first two crosses, while in third (*HKI1128*×*HKI193-1*) it was 1:1 (table 3). Background selection in the heterozygous plants using polymorphic SSRs led to the recovery of 84.9–93.9% RPG in *HKI323*×*HKI161*, 82.1–93.8% in *HKI1105*×*CML161* and 89.6–94.0% in *HKI1128*×*HKI193-1*. Two plants each in *HKI323*- (93.2 and 93.9% RPG) and *HKI1128*- (93.3 and 94.0% RPG), while three in *HKI1105*-derived progenies were advanced (82.1%, 91.0% and 91.1% RPG) (table 4).

***BC_2F_2 generation*:** Foreground selection identified 58 homozygous plants (*o*2/*o*2) in *HKI323*×*HKI161*, while the same was 20 and 60 in *HKI1105*×*CML161* and *HKI1128*×*HKI193-1*, respectively (table 3). All the three crosses deviated from the expected segregation pattern of 1:2:1 (table 3). Homozygous plants (*o*2/*o*2) with similarity to their respective recurrent parents were selected for advancement.

***BC_2F_4 generation*:** The highest recovery of RPG observed was 98.0% in *HKI323*×*HKI161*, 96.6% in *HKI1105*×*CML161* and 98.3% in *HKI1128*×*HKI193-1* (table 4). Based on higher recovery of RPG, phenotypic similarity to the recurrent parent and desirable degree of grain modification, *HKI323-44-68-16*, *HKI1105-22-99-3* and *HKI1128-48-1-14* were finally selected and used for advancement.

Evaluation of introgressed inbreds for grain quality and yield attributes

***Endosperm lysine and tryptophan in MAS-derived inbreds*:** Across years, 58.5–70.0% increase in lysine, and 46.0–96.0%

Table 2. Per cent polymorphism and distribution of SSRs used for background selection in the study.

Chromosome	*HKI323*×*HKI161*		*HKI1105*×*CML161*		*HKI1128*×*HKI193-1*	
	No. of markers screened	Polymorphic markers	No. of markers screened	Polymorphic markers	No. of markers screened	Polymorphic markers
1	43	16 (11)	43	18 (8)	44	14 (11)
2	37	15 (8)	37	18 (9)	49	20 (13)
3	40	24 (12)	40	24 (13)	46	29 (12)
4	36	12 (8)	36	15 (8)	49	19 (8)
5	32	20 (7)	32	11 (4)	33	16 (10)
6	27	19 (7)	27	11 (7)	38	22 (10)
7	42	14 (6)	42	19 (6)	40	21 (5)
8	30	9 (4)	30	8 (6)	45	13 (8)
9	38	9 (7)	38	20 (9)	50	25 (10)
10	26	8 (4)	26	10 (3)	39	7 (5)
Total	463	140 (74)	351	144 (73)	433	186 (92)
Percentage (%)	–	30.2	–	41.0	–	42.9

Values in parenthesis indicates the number of polymorphic markers used in background selection.

Table 3. Segregation of *o2* allele in each of the backcross and selfed progenies across the three crosses.

Cross	Generation	No. of plants genotyped	SSR used for genotyping	Genotypic class			χ^2	*P* value
				O2/O2	*O2/o2*	*o2/o2*		
HKI323×HKI161	BC_1F_1	154	*umc1066*	133	21	–	81.46	< 0.0001
	BC_2F_1	152		99	53	–	13.92	0.0002
	BC_2F_2	153		41	54	58	17.01	0.0002
HKI1105×CML161	BC_1F_1	153	*phi057*	121	32	–	51.77	< 0.0001
	BC_2F_1	161		104	57	–	13.72	0.0002
	BC_2F_2	155		69	66	20	34.40	< 0.0001
HKI1128×HKI193-1	BC_1F_1	130	*umc1066*	80	50	–	6.92	< 0.0085
	BC_2F_1	154		86	68	–	2.10	0.1469
	BC_2F_2	220		91	69	60	39.30	< 0.0001

enhancement in tryptophan was observed in the selected progenies over their respective recurrent parents (table 5). The concentration of protein in endosperm remained almost same in both original and introgressed inbreds. However, the protein quality was significantly improved in MAS-derived inbreds (table 5).

***Endosperm modification in introgressed progenies*:** For *HKI323*-based progenies, degree of opaqueness varied from 25–75%, while for *HKI1105* and *HKI1128*, it was 25–50% and 50–100%, respectively. However, the MAS-derived inbred that was selected for generating the hybrid combinations possessed 25% opaqueness for *HKI323-44-68-16* and 50% for each of *HKI1105*-22-99-3 and *HKI1128-HKI1128-48-1-14* (table 4).

***Morphological characteristics of MAS-derived inbreds*:** The MAS-derived inbreds showed high degree of resemblance with their respective recurrent parents (see table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/). However, the introgressed inbreds differed from their original inbreds for very few characters. For example, anthocyanin colouration in brace root and base of glume is present in *HKI1105*, while absent in QPM of *HKI1105* (*HKI1105-22-99-3*). The MAS-derived inbreds however possessed similar grain yield as achieved in original inbreds (table 6).

Evaluation of reconstituted hybrids for grain quality attributes, yield attributes and responses to diseases

***Endosperm lysine and tryptophan in MAS-derived hybrids*:** MAS-derived QPM version of *HM4* (*HKI1105-22-99-3×HKI323-44-68-16*), *HM8* (*HKI1105-22-99-3×HKI161*) and *HM9* (*HKI1105-22-99-3×HKI1128-48-1-14*), were designated as *HM4-Q, HM8-Q* and *HM9-Q,* respectively. The concentration of lysine and tryptophan in endosperm of reconstituted hybrids also recorded significant improvement over their respective original hybrids. Based on both the years, 50–78% enhancement in lysine was recorded, while tryptophan showed 51–100% increase across hybrids (table 7). Protein content remained almost same in both MAS-derived and original hybrids, but the protein quality showed significant enhancement. The increase in lysine in protein was in the range of 49.5 to 77.0%, while, the same for tryptophan in protein was 61.0 to 97.5% (table 7).

***Evaluation of MAS-derived hybrids for morphological characteristics*:** The reconstituted hybrids resembled their respective original hybrids with high degree of similarity except a few (see table 2 in electronic supplementary material). The selfed F_2 seeds of *HM4-Q*, *HM8-Q* and *HM9-Q* showed desirable degree of endosperm modifications. Grain yield

Table 4. Recurrent parent genome (RPG) recovery and degree of opaqueness in different backcross generations.

Cross	Generation	Genotype advanced	% RPG recovery	Range of RPG % in selected foreground positive plants	Opaqueness (%)
HKI323×HKI161	BC_1F_1	*HKI323-31*	73.6	63.3–74.3	–
		HKI323-44	74.3		–
	BC_2F_1	*HKI323-31-6*	93.2	84.9–93.9	–
		HKI323-44-68	93.9		–
	BC_2F_4	*HKI323-44-68-16*	98.0*	91.9–98.7	25
		HKI323-44-68-1	94.6		25–75
		HKI323-44-68-7	96.0		25–75
HKI1105×CMLI61	BC_1F_1	*HKI1105-22*	76.3	65.5–77.0	–
		HKI1105-24	77.0		–
	BC_2F_1	*HKI1105-22-14*	82.1	82.1–93.8	–
		HKI1105-22-99	91.0		–
		HKI1105-24-89	91.1		–
	BC_2F_4	*HKI1105-22-14-15*	92.0	82.6–96.6	25–50
		HKI1105-22-99-3	96.6*		50
		HKI1105-24-89-31	94.3		25–50
HKI1128×HKI193-1	BC_1F_1	*HKI1128-48*	90.0	80.0–91.9	–
		HKI1128-44	85.5		–
	BC_2F_1	*HKI1128-48-1*	94.0	89.6–94.0	–
		HKI1128-44-11	93.3		–
	BC_2F_4	*HKI1128-48-1-3*	96.2	91.9– 98.4	75
		HKI1128-48-1-14	95.1*		50
		HKI1128-48-1-16	98.4		75

*Used for generating hybrid combination contributed to national testing.

Table 5. Performance of original and improved inbreds for lysine and tryptophan in endosperm.

Trait	Year	*HKI323* (RP)	*HKI161* (DP)	*HKI323Q*	% Increase over *HKI323*	*HKI1105* (RP)	*CML161* (DP)	*HKI1105Q*	% Increase over *HKI1105*	*HKI1128*	*HKI193-1* (DP)	*HKI1128Q*	% Increase over *HKI1128*
% Lysine in sample	2013	0.170	0.336	0.277	63	0.195	0.373	0.341	75	0.194	0.321	0.317	63
	2014	0.181	0.311	0.304	68	0.226	0.398	0.373	65	0.223	0.330	0.344	54
% Tryptophan in sample	2013	0.039	0.071	0.067	72	0.052	0.079	0.076	46	0.044	0.077	0.080	82
	2014	0.035	0.073	0.067	91	0.049	0.082	0.079	46	0.039	0.080	0.082	110
% Protein	2013	8.40	9.70	9.00	–	9.90	10.20	9.80	–	12.00	9.3	11.70	–
	2014	8.50	9.80	8.80	–	9.70	10.10	9.70	–	11.70	9.2	11.40	–
% Lysine in protein	2013	2.02	3.46	3.07	52	1.97	3.66	3.48	95	1.62	3.45	2.71	67
	2014	2.13	3.17	3.45	62	2.33	3.94	3.85	69	1.90	3.59	3.02	59
% Tryptophan in protein	2013	0.46	0.73	0.74	61	0.53	0.77	0.78	47	0.37	0.83	0.68	84
	2014	0.41	0.75	0.76	86	0.51	0.81	0.81	59	0.33	0.87	0.72	118

HKI323-Q, *HKI323-44-68-16*; *HKI1105-Q*, *HKI1105-22-99-3*; *HKI1128-Q*, *HKI1128-48-1-14*.

Table 6. Agronomic performance of improved inbreds *vis-à-vis* original inbreds at Delhi during rainy season 2013 and 2014.

Genotype	Year of testing	MF (d)	FF (d)	PH (cm)	EH (cm)	TL (cm)	LBW (cm)	CL (cm)	CG (cm)	NR	NKR	100 KW (g)	YLD (kg/Ha)
HKI323	2013	49.0	51.0	137.5	72.2	30.0	7.1	12.7	3.2	11.7	19.2	24.8	2370
	2014	48.0	50.0	139.2	71.7	36.0	8.0	12.3	3.3	12.0	20.0	26.5	3015
HKI323Q	2013	51.5	53.5	130.0	64.2	32.2	8.4	13.3	3.5	13.3	18.7	24.4	2335
	2014	48.5	50.0	140.0	69.2	34.8	8.0	12.4	3.3	12.0	18.8	26.1	3389
HKI1105	2013	52.5	52.5	107.5	65.7	25.4	10.1	14.3	3.5	13.0	20.8	26.6	2096
	2014	51.0	51.5	114.2	75.0	31.3	10.2	14.3	3.4	13.0	21.0	25.2	2765
HKI1105Q	2013	49.5	51.0	100.0	50.8	25.4	9.8	13.8	3.3	12.0	19.8	24.5	1920
	2014	51.0	51.5	111.7	66.7	28.3	9.9	14.1	3.3	13.7	20.8	24.3	2795
HKI1128	2013	53.5	55.5	155.8	86.3	31.5	7.0	14.4	3.3	13.0	21.3	21.3	1595
	2014	51.0	51.0	162.5	88.3	36.5	7.4	14.8	3.3	12.7	22.2	23.1	3048
HKI1128Q	2013	51.5	53.5	165.0	84.2	33.4	7.8	15.3	3.1	12.7	21.3	22.4	1599
	2014	50.5	51.0	160.0	84.2	37.5	7.8	14.9	3.3	13.0	22.0	22.4	2959
SE	2013	0.72	0.72	10.72	5.56	1.45	0.55	0.38	0.07	0.26	0.46	0.78	142.97
	2014	0.57	0.28	9.03	3.59	1.48	0.49	0.48	0.02	0.27	0.53	0.68	93.63

HKI323-Q, *HKI323-44-68-16*; *HKI1105-Q*, *HKI1105-22-99-3*; *HKI1128-Q*, *HKI1128-48-1-14*; MF, days to 50% male flowering; FF, days to 50% female flowering; PH, plant height; EH, ear height; TL, tassel length; LBW, width of leaf blade; CL, cob length; CG, cob girth; NR, number of rows; NKR, number of kernel per row; 100 KW, 100 kernel weight; YLD, grain yield; d, days.

Table 7. Performance of original and improved hybrids for lysine and tryptophan in endosperm.

Quality attributes	Year	*HM4*	*HM4-Q*	% Increase over *HM4*	*HM8*	*HM8-Q*	% Increase over *HM8*	*HM9*	*HM9-Q*	% Increase over *HM9*
% Lysine in sample	2013	0.155	0.232	50	0.194	0.340	75	0.202	0.340	68
	2014	0.162	0.245	51	0.191	0.321	68	0.197	0.351	78
% Tryptophan in sample	2013	0.032	0.064	100	0.046	0.081	76	0.045	0.068	51
	2014	0.036	0.070	94	0.044	0.085	93	0.043	0.071	65
% Protein	2013	8.60	8.70	–	8.60	8.80	–	8.90	8.60	–
	2014	8.70	8.70	–	8.80	8090	–	9.0	8.90	–
% Lysine in protein	2013	1.80	2.67	48	2.26	3.86	71	2.27	3.95	74
	2014	1.86	2.81	51	2.17	3.61	66	2.19	3.94	80
% Tryptophan in protein	2013	0.37	0.74	100	0.53	0.92	74	0.51	0.79	55
	2014	0.41	0.80	95	0.50	0.96	92	0.48	0.80	67

HM4-Q, HKI1105-22-99-3×HKI323-44-68-16; HM8-Q, HKI1105-22-99-3×HKI161; HM9-Q, HKI1105-22-99-3×HKI1128-48-1-14.

Table 8. Agronomic performance of improved hybrids *vis-à-vis* original hybrids at Delhi during kharif 2013.

Genotype	MF (d)	FF (d)	PH (cm)	EH (cm)	TL (cm)	LBW (cm)	CL (cm)	CG (cm)	NR	NKR	100KW (g)	YLD (kg/Ha)
HM4	44.0	45.0	181.7	40.3	107.5	11.6	17.6	3.9	13.3	34.7	30.8	7160
HM4-Q	44.5	45.5	175.0	38.3	106.7	11.5	17.3	4.0	13.3	37.0	29.0	7042
HM8	45.0	45.0	158.3	36.7	93.3	11.0	19.1	4.6	14.0	34.8	32.2	6649
HM8-Q	46.0	46.5	167.5	36.2	93.3	10.9	18.9	4.6	14.3	37.5	30.3	6954
HM9	44.5	45.5	171.7	38.6	101.7	9.1	20.5	4.3	14.3	36.0	28.2	6347
HM9-Q	44.5	45.5	178.3	42.1	105.8	8.3	21.2	4.4	13.3	40.7	28.0	6084
SE	0.29	0.23	3.49	0.92	2.74	0.57	0.64	0.12	0.21	0.93	0.68	176.73

MF, days to 50% male flowering; FF, days to 50% female flowering; PH, plant height; EH, ear height; TL, tassel length; LBW, width of leaf blade; CL, cob length; CG, cob girth; NR, number of rows; NKR, number of kernel per row; 100 KW, 100 kernel weight; YLD, grain yield; d, days; SE, standard error.

and other contributing traits were also similar among the original and MAS-derived hybrids (table 8).

The data generated during 2014 and 2015 under the multilocation trials of AICRP-Maize, clearly suggested that QPM version of reconstituted hybrids showed high degree of resemblance to their original hybrids for grain yield and yield traits (see table 3 in electronic supplementary material). The flowering behaviour and maturity of the reconstituted hybrids were similar to the original hybrids as well (see table 3 in electronic supplementary material).

Further, the reconstituted and original hybrids also showed high degree of resemblance for resistance to important diseases of maize in the country (see table 4 in electronic supplementary material). For example, *HM4* during 2014 had the disease score of 3.0 against maydis leaf blight, while the same was 2.4 for *HM4-Q*. *HM8* and *HM9* recorded score of 3.0 and 2.8, while their QPM versions had 2.7 and 2.3, respectively. Similarly, all the hybrids recorded moderate resistance against turcicum leaf blight during 2015 (see table 4 in electronic supplementary material).

Discussion

MABB has been employed to introgress low phytic acid (*lpa*) (Naidoo *et al.* 2012), *o*2 (Gupta *et al.* 2013) and *crtRB1* (Muthusamy *et al.* 2014) alleles to improve nutritional quality traits in maize. In the present investigation, distinct marker polymorphism between the respective recurrent and donor parents facilitated the introgression of recessive *o*2 allele into the elite inbreds. Since we used *o*2-specific SSRs (located within the target gene), segregants could be selected with 100% efficacy (Babu *et al.* 2005). We also found severe segregation distortion (SD) for the *o*2 as reported by Jompuk *et al.* (2011). The reason for the SD could be the presence of many segregation distortion regions (SDRs) throughout the maize genome (Lu *et al.* 2002). Frequent occurrence of SD thus necessitates generation of large populations for obtaining sufficient numbers of foreground-positive plants (Muthusamy *et al.* 2014).

Introgression of *o*2 allele resulted in significant increase in protein quality by enhancing the concentration of lysine and tryptophan in the endosperm of both MAS-derived inbreds and hybrids. Lysine in protein recorded 48–95% enhancement, while tryptophan in protein showed 47–118% increase across inbreds/hybrids over their respective genotypes. The mechanism of enhancement of lysine and tryptophan in *o*2 genotypes are of diverse type. The enhancement of nutritional quality in *o*2 mutant is mainly due to (i) reduction of lysine deficient zein proteins followed by enhanced synthesis of lysine-rich non-zein proteins (Habben *et al.* 1993), (ii) reduced transcription of lysine catabolizing enzyme, lysine keto-reductase, (Kemper *et al.* 1999), and (iii) enhanced synthesis of various lysine-rich proteins and enzymes (Jia *et al.* 2013).

Though introgression of *o*2 allele alone has a major effect on accumulation of lysine and tryptophan in higher concentration, the levels of the same varied substantially across genetic background. For example, lysine ranged from 0.277 to 0.373%, while tryptophan varied from 0.067 to 0.082% across three inbreds. The variation is due to amino acid modifier loci that influence the accumulation lysine and tryptophan in *o*2 genetic background (Pandey *et al.* 2015). The levels of lysine and tryptophan in MAS-derived hybrids also showed large variation, and interactions of amino acid modifiers contributed by both the parents possibly determined the final level of the targeted amino acids. Further, the lysine and tryptophan levels were lesser in *HKI323-44-68-16* and *HKI1105-22-99-3*, compared to their respective donor parents (*HKI161* and *HKI193-1*, respectively). It is possibly due to the fact that favourable modifier loci present in the QPM donor was lost owing to repeated backcrossing to recurrent parent. QTLs for these modifier loci in QPM background have been identified recently, and can be used along with *o*2 allele in future breeding programme (Babu *et al.* 2015).

SSRs covering all 10 chromosomes were used for recovering the major proportion of the RPG within two backcross generations. The highest RPG recovery among the selected progenies was 96.58% in *HKI1105*×*CML161*, while it was 97.97% and 98.35% in *HKI323*×*HKI161* and *HKI1128*×*HKI193-1*, respectively. To achieve comparable results, conventional breeding would take five backcrosses since *o*2 is recessive in nature. In conventional method, BC_5F_3 progeny would be crossed with its other parent to reconstitute the hybrid. Thus, it would require 14 seasons from crossing the recipient and donor to the evaluation of hybrids. In contrast, MABB approach used here took 8–9 seasons since BC_2F_4 (*HKI323*- and *HKI1105*-based)/ BC_2F_5 (*HKI1128*-based) progenies were crossed to reconstitute the hybrids. The MABB approach thus clearly saved time of raising 5–6 additional seasons, and therefore accelerated the pace of breeding (Gupta *et al.* 2013; Muthusamy *et al.* 2014).

Phenotypic features such as plant, ear and grain characteristics used in combination with MAS helped to recover the RPG even rapidly (Manna *et al.* 2005; Muthusamy *et al.* 2014). Although, introgressed inbreds and reconstituted hybrids resembled their respective recurrent parents or original hybrids for majority of characters, they also differed for a few characters. In fact, morphological characteristics that show sharp contrast are highly useful for registration of genotypes (Gunjaca *et al.* 2008). Besides they also act as morphological marker to unambiguously differentiate the QPM-versions from the original inbreds during seed production and certification. The contrasting features are possibly due to the effects of minor proportion of donor genome (2.03–4.89%) present in the introgressed progenies (Choudhary *et al.* 2014).

The selected MAS-derived *o*2 inbreds across three genetic backgrounds showed desirable degree (25–50%

opaqueness) of endosperm modification. *CML161, HKI 161* and *HKI193-1* are the popular QPM inbreds and possess hard endosperm (50% opaqueness) due to the presence of favourable endosperm modifier loci (Paez 1973; Pandey *et al.* 2015). Although no marker system was used for the selection of endosperm modifiers in the backcross generations, the screening of kernels from *o*2/*o*2 plants on light box could successfully select the desirable progenies that possessed higher modification (Gupta *et al.* 2013).

The grain yield potential of the reconstituted hybrids was *at par* with the original hybrids across multiple locations. Selection of SSR alleles specific to recurrent parents indirectly led to the selection of unknown loci associated with yield, agronomic traits and heterosis (Gupta *et al.* 2013; Muthusamy *et al.* 2014). Further, a similar response recorded in reconstituted and original hybrids against various diseases is also due to similar genomic constitution achieved through high recovery of RPG. Here also, the loci involved in responses to various diseases were not selected in the backcross progenies, but the background selection helped in retaining those unknown loci. However, in some cases the responses in QPM versions were slightly different from the original hybrids. These minor changes in response to diseases are due to the presence of minor fragments of the donor parent genome. QPM versions of *HM4*, *HM8* and *HM9* have now been released and notified for commercial cultivation in their respective zones. The newly developed QPM hybrids with better protein quality, high grain yield and diverse adaptation offer promise in reducing nutritional insecurity.

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