Indian Journal of Agricultural Sciences **88** (2): 253–9, February 2018/Article

Haplotypes of recessive *opaque2* allele in exotic- and indigenous-quality protein maize inbreds

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Received: 12 July 2017; Accepted: 24 October 2017

ABSTRACT

Forty six QPM inbreds of diverse origin were genotyped using *opaque2* (*o2*)-specific SSRs viz. *umc1066* and *phi057*, present on exon-1 and exon-6 of the gene, respectively. These SSRs are the polymorphic sites that differentiate dominant (*O2*) and recessive (*o2*) alleles. A total of two alleles (*o2*-A and *o2*-B) of *umc1066*, and three alleles (*o2*-C, *o2*-D and *o2*-E) of *phi057* were identified. *o2*-A allele (93.48%) was the most abundant, while *o2*-B allele was observed only in 6.52% of the inbreds. For *phi057* locus, *o2*-E allele had the highest frequency (80.17%), followed by *o2*-D (19.57%) and *o2*-C (2.17%). Among various haplotypes, *o2*-AE had the highest frequency (76.09%), followed by *o2*-AD (15.22%) and *o2*-BD (4.35%). *o2*-BC and *o2*-AC haplotypes were observed in 2.17% each of the inbreds. Haplotype, *o2*-BE was not found in the inbred panel. Cluster analyses grouped the inbreds into two major clusters with inbreds being together based on haplotypes. All the inbreds developed at PJTSAU, Hyderabad, CSK HPKV, Bajaura, and CCS HAU, Uchani and majority of inbreds from CIMMYT, Mexico had the *o2*-AE haplotype. Inbreds developed at VPKAS, Almora had three haplotypes viz. *o2*-BC, *o2*-AD and *o2*-AE. Inbreds from IARI, New Delhi had *o2*-AE and *o2*-AD haplotypes, while inbreds developed at IIMR, New Delhi had *o2*-AE and *o2*-AC haplotypes. *o2*-BD was identified as the most promising haplotype for accumulation of lysine and tryptophan in the endosperm, followed by *o2*-AE. *o2*-BC haplotype was the least promising for nutritional quality. *o2*-AC, *o2*-AD and *o2*-BC possessed 25% opaqueness, while *o2*-BD had 50% opaqueness. Broad range of 25-100% opaqueness was observed among inbreds possessing *o2*-AE haplotype. So far, breeders perceive that only one recessive *o2* allele exist in the population, and any QPM inbred serves as a donor of the *o2* allele in the QPM breeding programme. The present study reports here the occurrence of at least five versions of recessive *o2* allele, and the information generated here can benefit the QPM breeding programme by selectively introgressing the most favourable haplotype allele of *o2* over other haplotypes.

Key words: Cluster analyses, Exon, Haplotype, *Opaque2*, QPM, SSR

Malnutrition caused due to inadequate consumption of unbalanced diet has emerged as one of the alarming problems in the under-developed and developing world (Bouis *et al.* 2011). While an estimated two billion people suffers from malnutrition, 795 million people are undernourished across the globe (IFPRI 2016a). Among various types of malnutrition, protein energy malnutrition (PEM) is considered to be one of the most lethal forms. Inadequate intake of both protein and calories leads to '*marasmus*', whereas fair-to-normal calorie intake with inadequate protein intake causes '*kwashiorkor*' (Bain *et al.* 2013). Further consumption of unbalanced protein restricts proper growth and development in human. It is estimated that alleviating malnutrition is one of the most cost-effective steps with every $1 invested in proven nutrition programme offers benefits worth $16 (IFPRI 2016b). Thus efforts directed towards providing the balanced and nutritious food assumes great significance (Gupta *et al.* 2015a, Yadav *et al.* 2015, Hossain *et al.* 2017).

Among various cereals, maize serves as an important source of food, and together with rice and wheat, it provides at least 30% of the food calories to more than 4.5 billion people in 94 developing countries (Shiferaw *et al.* 2011). The demand for cereals will continue to increase as a consequence of the expanding human population. The world will have around 7.7 billion people by 2020, and it will reach up to 9.3 billion by 2050. The demand for maize between now and 2050 will double in the developing world (Rosegrant *et al.* 2009). Traditional maize contains 8-10% protein in the endosperm, but it's deficient in two essential amino acids such as lysine and tryptophan, whose amount is less than one-half of the concentration recommended for human nutrition (Gupta *et al.* 2015b). Non-ruminant animals, including humans, have specific requirements for each of the essential amino acids, and healthy diets must include alternate source of these amino acids. The discovery of nutritional value of the *opaque2* (*o2*) mutation in maize

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was a significant breakthrough, as it alters the amino acid composition of the endosperm protein, resulting in nearly two-folds increase in lysine and tryptophan (Mertz *et al.* 1964). Successful utilization of *o2* coupled with endosperm modifier loci led to the birth of 'Quality Protein Maize' (QPM) (Vasal *et al.* 1980). Several countries in Asia, Africa and Latin America have released diverse QPM cultivars adaptable various agro-ecologies using conventional breeding approaches (Gupta *et al.* 2015b). The recessive *o2* allele has also been introgressed into elite normal inbreds through molecular- breeding approaches (Babu *et al.* 2005, Gupta *et al.* 2013 & 2015b, Jompuck *et al.* 2011). The cloning and characterization of the *o2* gene (Schmidt *et al.* 1987, Motto *et al.* 1988), followed by detection of two SSRs (*phi057* and *umc1066*) within the gene led to effective differentiation of the dominant (*O2*) and recessive (*o2*) alleles (Yang *et al.* 2004). Among SSRs present within *o2* gene, *umc1066* is present on exon-1, while *phi057* is located on exon-6 of the gene (Yang *et al.* 2004). These SSRs are codominant in nature, and used in molecular breeding programme as they differentiate homozygote from heterozygote in backcross- and selfed- generations of the marker-assisted breeding programme (Babu *et al.* 2005). The SSR, *phi112* present between G box and 3 upstream open reading frames in the leader sequence (Schmidt *et al.* 1990), was not considered in the present analyses as it is dominant in nature, and is not used in the molecular marker-assisted programme (Gupta *et al.* 2013). Breeders generally perceive that only type of recessive *o2* allele exist in the QPM germplasm, as all QPM inbreds leads to the enhancement of lysine and tryptophan in the endosperm. Molecular characterization of *o2* allele at the two SSR loci present within the gene would provide the insight of the allelic variation. So far information on different alleles of *o2* based on the SSRs present within the gene is not available to the breeders. The present study was therefore undertaken to analyze the presence of different forms of recessive *o2* allele present among diverse QPM inbreds, and (ii) study the effects of different *o2*, if any, on accumulation of lysine and tryptophan in the endosperm.

MATERIALS AND METHODS

Forty six QPM inbreds of diverse pedigree were selected in the presented study (Table 1). These inbreds differ for their endosperm amino acid concentration and kernel hardness (Pandey *et al.* 2015). The inbreds include 39 QPM inbreds of Indian origin; out of which ten are developed by PTJSAU, Hyderabad; nine from ICAR-IARI, New Delhi; seven from CSK-HPKV, Bajaura; five from ICAR-VPKAS, Almora; five developed under the CCS HAU, Uchani; and three from the ICAR-IIMR, Ludhiana. Seven exotic QPM inbreds developed by CIMMYT, Mexico were also selected for this study.

Leaf samples were collected from three weeks old individual seedlings (3-4 leaf stage) and DNA was extracted from the leaves using standard CTAB method with minor modifications (Saghai-Maroof *et al.* 1984) optimized at Maize Genetics Unit, Division of Genetics, IARI. The isolation, quantification and PCR amplification was carried out as per the procedure described in Muthusamy *et al.* (2014). PCR amplicons were separated in 4.5% superfine agarose using horizontal gel electrophoresis.

Scoring of the SSR alleles was performed manually with respect to positions of the bands relative to the 100 bp ladder which was run parallel with the inbred lines. Finally alleles were numbered sequentially from the smallest to the largest sized products to analyze their frequencies. Considering the number of alleles generated in each of SSR locus, different haplotypes were constructed for the *o2* allele. Pair-wise genetic similarity matrix between the genotypes based on SSR data was computed using Jaccard's genetic similarity coefficient, and the similarity matrix was further subjected to an agglomerative hierarchical classification by employing UPGMA (Unweighted Pair Group Method using Arithmetic Averages) clustering algorithm, using NTSYS-pc Ver. 2.11 a.

RESULTS AND DISCUSSION

In the present study, the frequency and distribution of DNA polymorphisms at *o2*-gene based justified viz., *umc1066* and *phi057* among the 46 QPM inbreds was examined. *umc1066* generated two alleles namely *o2*-A and *o2*-B; with 150 bp and 170 bp amplicons, respectively. 43 inbreds had the *o2*-A allele, while three inbreds had the *o2*-B allele (Table 1). Thus, *umc1066*-based *o2*-A allele (93.48%) was the most abundant, while *o2*-B allele could be observed only in 6.52% of the inbreds. SSR, *phi057* generated three alleles namely, *o2*-C (150 bp), *o2*-D (160 bp) and *o2*-E (170 bp), respectively. Two inbreds possessed *o2*-C allele, while nine and 35 inbreds had the *o2*-D and *o2*-E allele, respectively. *o2*-E allele therefore had the highest frequency (76.09%), followed by *o2*-D allele (19.57%), with *o2*-C allele being the least frequent (4.34%). Replication slippage and unequal crossing over are the primary mechanisms for producing new alleles at the SSR loci. Yang *et al.* (2004) while studying the genetic diversity at the *o2* locus, reported only one allele at *umc1066* locus, and two alleles at *phi057* locus among *o2* inbreds of Chinese- and CIMMYT- origin.

Among the six possible haplotypes namely, *o2*-AC, *o2*-AD, *o2*-AE, *o2*-BC, *o2*-BD and *o2*-BE, only five haplotypes were observed. Earlier, 10 versions (*o2-R*, *o2-m[r]*, *o2-Columbian*, *o2-Agroceres*, *o2-261*, *o2-mh*, *o2-33*, *o2-Go2-Charentes*, *o2-Italian*, and *o2-Crow*) of recessive (*o2*) alleles was detected by Southern analysis using two molecular probes corresponding to the 5′ and the 3′ end of the *O2* cDNA (Hartings *et al.* 1995a & b). However, due to radioactivity and elaborate assay, the southern based analysis is no longer in common use. On the other hand, SSR is a simple and easy to use PCR based marker system, and the popular choice of marker-assisted breeding programme. Further, the two SSRs are the polymorphic sites that differentiate the dominant- and recessive- *o2* allele. Thus characterization of *o2* using these two SSRs holds significance. It is interesting to note that in the present study, *o2*-BE haplotype could not be detected in the inbred panel.

Table 1 Details of QPM inbreds used for allelic diversity of *opaque*2 gene

Inbred lines	Pedigree	Source	Haplotype	*Tryptophan (%)	*Lysine (%)	*Opaqueness (%)
BQPML-5244	G33QC20	PJTSAU, Hyderabad	AE	0.111	0.444	100
BQPML-63-1-3	P61C1	PJTSAU, Hyderabad	AE	0.091	0.365	25
BQPML-5122	587(PC65Q)	PJTSAU, Hyderabad	AE	0.105	0.420	75
BQPML-5204-2-5	P65C6	PJTSAU, Hyderabad	AE	0.108	0.433	50
BQPML-5204-1-5	P65C6	PJTSAU, Hyderabad	AE	0.098	0.394	100
BQPML-5207-4-2	P66CO	PJTSAU, Hyderabad	AE	0.104	0.417	100
BQPML-10-1-1	G17QC8	PJTSAU, Hyderabad	AE	0.095	0.381	50
BQPML-199-2	G26QC23	PJTSAU, Hyderabad	AE	0.078	0.312	75
BQPML-62	P61C1	PJTSAU, Hyderabad	AE	0.076	0.306	50
BQPML-412	P66C0	PJTSAU, Hyderabad	AE	0.100	0.399	75
CML161	G25QC18H520	CIMMYT, Mexico	BD	0.094	0.376	50
CML162	G25QC1F18	CIMMYT, Mexico	AE	0.081	0.323	25
CML169	G26QC22H7	CIMMYT, Mexico	AE	0.081	0.324	25
CML170	G26QC22H9	CIMMYT, Mexico	AE	0.081	0.326	25
CML173	P68C1F180	CIMMYT, Mexico	AE	0.099	0.395	25
CML176	P63-12-2-1/P67-5-1-1	CIMMYT, Mexico	AE	0.081	0.326	25
CML180	G32Q/EV8444SRBC4	CIMMYT, Mexico	AD	0.087	0.349	25
LQPM-2	S0/SN Comp(P)SN6	CSK-HPKV, Bajaura	AE	0.095	0.381	75
LQPM-10	28FSF(MS)HEC	CSK-HPKV, Bajaura	AE	0.087	0.350	75
LQPM-19	CIMMYT population-6482	CSK-HPKV, Bajaura	AE	0.090	0.361	75
LQPM-20	S0/SN Comp	CSK-HPKV, Bajaura	AE	0.098	0.390	100
LQPM-30	28FS(MS)HEC	CSK-HPKV, Bajaura	AE	0.080	0.319	75
LQPM-34	Shakti(S0)HE25	CSK-HPKV, Bajaura	AE	0.097	0.389	50
LQPM-40	CIMMYT population-6482	CSK-HPKV, Bajaura	AE	0.081	0.325	25
VQL1	CM212-*o2*	VPKAS, Almora	BC	0.056	0.223	25
VQL2	CM145-*o2*	VPKAS, Almora	AD	0.064	0.255	25
VQL5	V25-*o2*	VPKAS, Almora	AD	0.063	0.254	25
VQL8	CM145-*o2*	VPKAS, Almora	AE	0.089	0.357	100
VQL26	V351-*o2*	VPKAS, Almora	AE	0.097	0.389	50
DMRQPM-60	28 FS (MS)6 HECC	IIMR, Ludhiana	AC	0.081	0.326	25
DMRQPM-03-102	Derivative of 'Shakti'	IIMR, Ludhiana	AE	0.084	0.336	75
DMRQPM-121	Derivative of 'Shakti'	IIMR, Ludhiana	AE	0.085	0.338	100
HKI161	Selection from CML161	CCS-HAU, Uchani	BD	0.090	0.362	50
HKI163	Selection from CML163	CCS-HAU, Uchani	AE	0.082	0.326	50
HKI170	Selection from CML170	CCS-HAU, Uchani	AE	0.080	0.321	25
HKI193-1	Selection from CML193	CCS-HAU, Uchani	AE	0.085	0.341	75
HKI193-2	Selection from CML193	CCS-HAU, Uchani	AE	0.085	0.341	100
MGUQ-101	HKI1105-*o2*	IARI, New Delhi	AE	0.078	0.312	50
MGUQ-102	HKI1128-*o2*	IARI, New Delhi	AE	0.090	0.360	50
MGUQ-103	HKI323-*o2*	IARI, New Delhi	AD	0.080	0.318	25
MGUQ-104	CM137-*o2*	IARI, New Delhi	AE	0.078	0.314	25
MGUQ-105	CM138-*o2*	IARI, New Delhi	AE	0.077	0.309	25
MGUQ-106	CM139-*o2*	IARI, New Delhi	AD	0.089	0.357	25
MGUQ-107	CM140-*o2*	IARI, New Delhi	AD	0.087	0.348	25
MGUQ-108	CM150-*o2*	IARI, New Delhi	AE	0.076	0.304	25
MGUQ-109	CM151-*o2*	IARI, New Delhi	AD	0.075	0.300	25
Mean				0.086	0.346	51.63
SED				0.002	0.006	4.141

*As per Pandey *et al.* (2015)

This haplotype is possibly the least favoured, and present in very less frequency in the germplasm. With the inclusion of more QPM lines from diverse sources, *o2*-BE haplotype may be detected. Among the haplotypes observed in the present study, *o2*-AE was found in 35 inbreds, followed by *o2*-AD (seven inbreds), *o2*-BD (two inbreds), *o2*-BC (one inbred) and *o2*-AC (one inbred) (Table 1). Thus, *o2*-AE had the highest frequency (76.09%), followed by *o2*-AD (15.22%) and *o2*-BD (4.35%). *o2*-BC and *o2*-AC haplotypes had the frequency of 2.17% each. All the inbreds developed at PJTSAU, Hyderabad, CSK-HPKV, Bajaura possess *o2*-AE haplotype. Majority of inbreds developed at CIMMYT, Mexico (except CML161 and CML180) and CCS-HAU, Uchani (except HKI161) also had the *o2*-AE haplotype. Haplotype *o2*-BD is found in one inbred each from CIMMYT, Mexico (CML161) and CCS-HAU, Uchani (HKI161). It is noteworthy to mention here that HKI161 and HKI170 derived from CML161 and CML170, respectively, possessed similar haplotype, indicating the inheritance of same *o2* allele during the selection process. Among various QPM breeding centres, inbreds developed at VPKAS, Almora had three haplotypes *viz.,* *o2*-BC, *o2*-AD and *o2*-AE, suggesting availability of diverse *o2* allele in their QPM germplasm. Inbreds from IARI, New Delhi had *o2*-AE and *o2*-AD haplotypes, while, inbreds developed at IIMR, Ludhiana had *o2*-AE and *o2*-AC haplotypes.

Cluster analyses grouped the inbreds into two major clusters. Cluster A was the largest cluster having 43 inbreds. All the *o2*-AE haplotypes were in sub-cluster A1, while *o2*-AC and *o2*-AD belonged to sub-cluster A2 and A3, respectively. Cluster B had three inbreds with *o2*-BD (sub-cluster B1) and *o2*-BC (sub-cluster B2) haplotypes. The genetic relationship among the inbreds based on the *o2*-haplotypes has been depicted in Fig. 1.

QPM inbreds with *o2* allele possess higher tryptophan and lysine in the endosperm as compared to normal maize (Hossain *et al.* 2007). In the present study, inbreds with *o2*-BD haplotype possessed the highest mean for tryptophan (0.092%) and lysine (0.369%); whereas haplotype *o2*-BC had the lowest mean for tryptophan (0.056%) and lysine (0.223%) (Table 1). Inbreds having *o2*-AE haplotype obtained a mean of 0.089% for tryptophan and 0.355% for lysine. *o2*-AC and *o2*-AD had moderate tryptophan (0.081% and 0.078%, respectively) and lysine (0.326% and 0.312%, respectively) concentration. Based on mean, *o2*-BD haplotype was the most promising for lysine and tryptophan, followed by the most abundant haplotype, *o2*-AE. *o2*-BC haplotype was the least promising for nutritional quality. The variation in accumulation of tryptophan and lysine is possibly due to function of different *o2* alleles. The *o2* gene

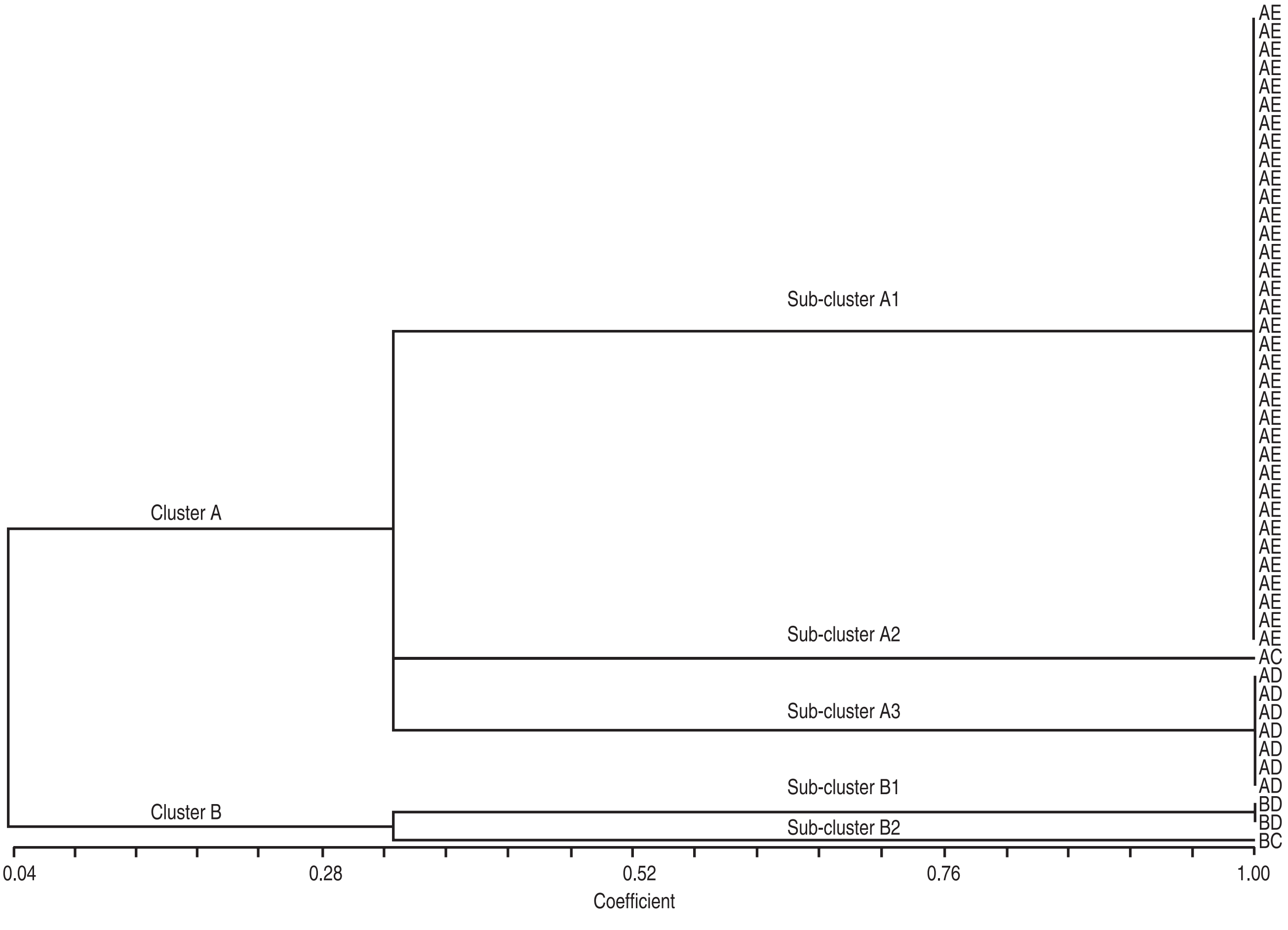

Fig 1 Dendogram depicting the genetic relationship among inbreds based on *o2*-haplotypes. Value on X-axis represents Jaccard's similarity coefficient. AC, AD, AE, BC and BD are the *o2*-haplotypes.

located on chromosome 7L produces leucine-zipper (bZIP) protein acts as a transcriptional factor for expression of zein family of storage protein genes, especially 22-kDa α-zeins (Ueda *et al.* 1992). The mutant protein causes reduction in synthesis of zein protein by 50-70% primarily due to its less affinity of binding to the promoter regions (Kodrzycki *et al.* 1989). The enhancement of nutritional quality in *o2* mutant is mainly due to reduction of lysine deficient zein proteins followed by enhanced synthesis of lysine-rich non-zein proteins (Habben *et al.* 1993). Recessive *o2* significantly also reduces transcription of lysine keto-reductase, the enzyme that degrades lysine in maize endosperm, thereby enhancing the concentration of lysine (Kemper *et al.* 1999). Further, *o2* is involved in regulation of various metabolic pathways and causes enhanced synthesis of various lysine-rich proteins and enzymes (Jia *et al.* 2013). Therefore, change in extent of function in *o2* alleles would likely to cause differential accumulation of tryptophan and lysine. In fact, change in nucleotide sequence at two exons increase or decrease the number of proline residues in the *O2* protein that influences the direction and degree of turns in the three-dimensional structure of the protein, and in turn affects the activity of the *O2* protein (Yang *et al.* 2004). However, variation for lysine and tryptophan within each of the haplotype is possibly due to amino acid modifier loci present in the genetic background (Babu *et al.* 2015, Gutierrez-Rojas *et al.* 2010, Pineda-Hidalgo *et al.* 2011).

In case of kernel opaqueness, *o2*-AC, *o2*-AD and *o2*-BC possessed 25% opaqueness, while BD had 50% opaqueness. Broad range of 25-100% opaqueness was observed among inbreds possessing AE haplotype. It is well understood that *o2* allele induces opaqueness. The reduction of α-zein synthesis in *o2* causes severe reduction in size of protein bodies (PBs) that in turn causes loose packaging with air spaces in between, leading to opaque- and soft- kernel (Wu *et al.* 2010). Favourable combination of endosperm modifier loci restores the kernel hardness. Two to three folds increase in 27-kDa γ-zein has been identified as the major factor of endosperm modification (Geetha *et al.* 1991). Elevated synthesis of 27-kDa γ-zein results in more PBs that is smaller in size (Lopes and Larkins 1995). Cystein residues in the periphery are engaged in formation of disulphide bonds with the neighbouring PBs that result in formation of compact packing thus vitreousness is restored (Wu *et al.* 2010). Besides 27-kDa γ-zein, several endosperm modifier loci affecting the kernel hardness have been identified (Holding *et al.* 2008, 2011). Amorphous, non-crystalline amylopectin molecules at the surface of starch granules in the modified kernel interact and form contacts that link starch granules together and restores hard and vitreous phenotype (Gibbon *et al.* 2003, Jia *et al.* 2013). In QPM breeding, opaqueness of 25-50% is generally favoured as higher degree of opaqueness makes the kernel more soft, and in turn lowers the density of grain and yield potential (Vivek *et al.* 2008). In all the *o2*-haplotypes, favourable degree of kernel modification was observed. Thus favourable combination of endosperm modifier loci in any of the *o2*-haplotypes would produce hard endosperm QPM genotypes (Hossain *et al.* 2008 a & b).

So far, it is perceived by the breeders that only one recessive *o2* allele is present in the population, and in homozygous state (*o2o2*) it possesses high lysine and tryptophan as compared to *O2O2* or *O2o2*. The present study reports here the occurrence of at least five haplotypes of recessive *o2* allele among available QPM germplasm. Though all the five haplotypes enhance the lysine and tryptophan, the extent varies thereby suggesting the role of differential effects of these haplotypes. It is also noteworthy to mention that a QPM hybrid may possess *o2* alleles with two different haplotypes contributed by its parental inbreds. For example, one inbred may possess the the most favourable haplotype (*o2*-BD), and other may have the least favoured haplotype (*o2*-BC). Thus the enhancement in lysine and tryptophan in their hybrid would be less than the potential value. Thus, two inbreds may be bred in a way that they possess the most favourable haplotype (*o2*-BD), and the hybrid thus possessing *o2*-BD haplotype in homozygous condition would effect the highest level of enhancement in lysine and tryptophan. To best of our knowledge this is the first report of availability of different *o2* haplotype alleles in the QPM germplasm.

The present study thus reports here the presence of five haplotypes of recessive *opaque2* allele among diverse Quality Protein Maize (QPM) inbreds of indigenous and exotic origin. Among various haplotypes, AE was the most predominant, and was present in almost two third of the inbreds. BC and AC were the least frequent ones, while BE was not found in the inbred panel. BD haplotype was the most promising for endosperm lysine and tryptophan, while BC haplotype was the least favoured.

ACKNOWLEDGEMENT

Financial support provided by IARI, New Delhi is duly acknowledged. We thank breeders of different breeding centres of AICRP-Maize, India and CIMMYT, Mexico for sharing their inbred.

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