Detection of Heterozygous *o2O2* Allele in BC_1F_1 Progeny of QPM Versus Normal Cross in Maize Using Marker-Assisted Selection

ABSTRACT

Marker assisted back crossing is reliable tool in selecting the heterozygotes in backcross generations. It quickens the selection process which would otherwise require an extra generation/season for raising the BCF_2 seed for finding the recessive allele which needs to be phenotypically expressed. Therefore in the present study normal maize (BML-6and IML-187) and QPM donor lines (DQL-2029-1 and DQL-779-2-9) were genotyped at *o2* locus using co-dominant SSR marker *phi057*. The marker allele size at 02 locus for normal parents was 155 bp in IML-187(P_1) and 160 bp in BML-6(P_2). 170bp in DQL-2029-1(P_3) and DQL-779-2-9(P_4) for QPM or donor parents. Based on this polymorphism crosses were made to generate F_1 hybrids. IML-87 was crossed with DQL-2029-1 and BML-6 was crossed with DQL-779-2-9. Thus, based on polymorphism at the DNA level, biparental backcrosses were made to eventually combine *o2* allele in the endosperm of normal maize. The two BC_1F_1 populations were genotyped at the *opaque-2* locus using *phi057* SSR marker. It was observed that out of 96 plants analyzed, 40 individuals were heterozygous at *o2* locus in BC_1F_1 population derived from the cross BC_1F_1 (A) while 43 out of the 100 plant analyzed in cross BC_1F_1 (B) were heterozygous when screened with *phi057* SSR marker. Marker Assisted backcrossing is a useful tool in trait introgression as it helps in pre-flowering elimination of negative plants as well as donor genome and need for selfing is eliminated in BCF_1s. SSR *phi-057* was able to clearly distinguish between QPM and normal parents and detect heterozygous plants in the backcross progeny.

Keywords: SSR; marker assisted selection; marker assisted backcrossing; heterozygote; maize.

1. INTRODUCTION

There is a nutritional challenge to the maize consumers, be it animals or humans because it is deficient in two important amino acids viz. lysine and tryptophan. Lysine content (1.6-2.7%) in maize protein is well below the recommendation by FAO (WHO/ FAO/ UN, 1985) for human. Although its content (5.4%) is adequate in germ protein, the abundance of endosperm protein 'zeins' (average lysine content ~1.9%); which comprises of 60–70% endosperm protein; reduces the overall level of lysine in the grain [1]. Similarly, the lack of tryptophan residues in zeins causes low level of tryptophan in endosperm protein (0.2-0.6 %). Amino acids are required in a specific ratio and lower levels of lysine or tryptophan affects the ability of body to use other amino acids. These amino acids are called limiting amino acids and their deficiency lead to reduced appetite, delayed growth, impaired skeletal development and aberrant behaviour [2,3]. A maize kernel consist of four protein types viz. albumins, globulins, zein and glutelin out of which zein consists 50–70% of total proportion [4]. The zein fractions are rich in cysteine and methionine amino acids and it also consists of glutamine, leucine and proline. It is completely devoid of lysine and tryptophan, whereas other proteins consist of these amino acids in large quantities. Zein is a class of prolamin proteins that are mainly present in maize. Improvement of protein quality of maize has been made possible through a mutant gene called opaque-2 (o2), which leads to the development of quality protein maize (QPM) [5]. Several natural mutants which confer high lysine and tryptophan levels had been identified in the 1960s and 1970s, these are opaque-2, opaque-6, opaque-7, floury-2 and floury-3 [6]. The

recessive *o2* mutant has been successfully utilized in the breeding programme for enhancement of protein quality [7]. Initially maize cultivars with *o2* mutation was not preferred by farmers and consumers due to soft and opaque endosperm, increased susceptibility to insect-pest, diseases, and breakage of grains during mechanical processing [8]. Endosperm modifier genes that confer hard endosperm in the *o2* background were introgressed at CIMMYT, Mexico [8] and University of Natal, South Africa [9]. This eventually led to the development of nutritionally enriched hard endosperm maize, popularly phrased as 'Quality Protein Maize' [7]. Worldwide different agricultural research centers have achieved significant progress in increasing the lysine and tryptophan content in the whole grain [10]. Maize varietal improvement and QPM conversion programs, a multi-trait selection procedure using independent selection levels have been employed to increase grain yield, resistance to pest and diseases, accumulate modifiers and improve other important traits in which QPM germplasm is defective. In QPM breeding program, protein and tryptophan analysis in germplasm is an important step [11]. A broad range of the CIMMYT maize populations have been converted to QPM. This germplasm is reported to have high potential for QPM cultivar development [12]. QPM with high protein quality and grain yield could be accepted by the farmers [6]. QPM germplasm has been widely used for the development of QPM cultivars with high grain yield in African countries [10]. The serious problem in QPM breeding is abiotic stresses. Water stress and soil infertility are the most important stresses that reduce maize productivity in developing countries. It inflicts major yield losses in maize in African countries [13]. Global climate change not only influences the soil fertility and water holding capacity of soil, but also affects the maize production [14].

A large number of normal maize hybrids have been released and commercialized worldwide. But the QPM-based germplasm is quite low, and very small number of genetically diverse QPM hybrids is available. Nearly 12 QPM hybrids have been released in India compared to more than 100 normal maize hybrids [15]. In this context, it is necessary to develop various QPM varieties not only in India but also in the world. Conversion of normal maize inbred parents of established into QPM hybrids requires lesser time due to tested combining ability, heterosis and adaptability of the released hybrids [16], but through conventional backcross breeding, the o2 recessive allele is not visible in BCF_1 generations due to non expression. So in a conventional crossing program, we need to self the backcross progeny which is half heterozygote and half dominant homozygotes (1:1), so that after seed set, when the cobs have a segregation ratio of 1:2:1 at O2 locus we might be able to detect homozygotes by biochemical analysis of lysine and tryptophan because a spiked value of 0.075% tryptophan in sample will be available only in a recessive homozygote. These recessive homozygotes are then sown in the next season for further backcrossing and so on. The problem in this system is difficulty in handling of such a large population and enlarged breeding time. But what if we are able to detect heterozygotes in the first place soon after first backcross generation seed is sown at seedling or vegetative stage. Molecular markers can surely achieve this feat thus enabling us to rogue out dominant homozygotes from the plant populations. There are three gene based SSR markers *phi 057, phi 112* and *umc 1066* which are closely associated with opaque locus can be used to differentiate between QPM and non QPM lines. In the present study we used these markers to differentiate between QPM and non QPM parents as well as heterozygotes and homozygotes because of co-dominant nature of these SSRs.

2. MATERIALS AND METHODS

The present investigation was carried out at Division of Genetics and Plant Breeding and at Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Faculty of Agriculture, Wadura Campus Sopore. Normal maize inbred lines namely IML-187 (IIMR-Ludhiana) and BML-6 (PJTSAU-Hyderabad) were used as recipient (recurrent) parents while DQL-2029-1 and DQL-779-2-9 developed by IIMR were used as QPM donor parents (donor) for *opaque-2* allele. The BML-6 and IML-87 are the parents of Shalimar Maize hybrid-5 also known as DMRH-1417. This is a very excellent medium maturity hybrid (120 days), with a yield potential of 8.5 t/ha with excellent tip filling and median cob placement. The above said inbred lines were analyzed for polymorphism with *opaque-2* gene specific SSR markers at the *opaque-2* locus. The crossing scheme is presented in the Fig. 1. DNA extraction was done using Qiagen DNA extraction kit and DNA was stored at -20^{o}C. PCR was done by using *Gotaq Green* master mix (12.5 ul) and 1 ul forward and 1 ul Reverse primer with 2 ul DNA was added to the PCR microtube and volume was raised to 25 ul per sample by adding 8.5 ul Millipore water. PCR reaction was done for 35 cycles of

annealing as mentioned in Table 2. Gel electrophoresis was done using 3% agarose gel run at 110V for 1 hour and 20 minutes alongwith 100 bp ladder (Fig. 2). Sequence of three SSRs *phi 057, phi 112* and *umc 1066* was downloaded from www. mgdb.org and is presented in Table 1. The backcross generation involving IML-187 as recurrent parent as marked as BC_1F_1 (A), whereas that involving BML6 is designated as BC_1F_1 (B). The PCR reactions involving BC1F1 plants from this biparental crossing scheme were also done using the similar procedure. Chi square ratios were also worked out for both the crosses.

3. RESULTS AND DISCUSSION

3.1 Marker Polymorphism at *O2* Locus

Three gene based SSR markers that are known to be linked with *o2* locus namely, *Phi057*, *phi112* and *umc1066* were used to detect the polymorphism between the parents used in the conversion program at o2 locus. In the present study, we observed that *phi112* marker exhibited dominant behavior between QPM and non-QPM lines. The allele found due to *phi112* primer was only observed in normal maize as it is *O2O2* and was absent in QPM lines. Therefore, this marker cannot be used in a conversion program but it is usable in determining the purity of QPM varieties. The markers *phi057* and *umc1066* exhibited co dominant polymorphism between IML-187, BML6 and DQL-2029, 2029-1. The marker *phi057* amplified approximately at 155 bp in IML-187(P_1) and 160 bpBML-6(P_2) whereas it amplified approximately around 170bp in DQL-2029-1(P_3) and DQL-779-2-9(P_4) (Table 3 and Fig. 2) and the occurrence of polymorphic alleles at *o2* locus clearly distinguishes these lines from one another on the basis of opaque gene. Therefore facilitates the use of *phi 057* marker in identifying the desired *o2* individuals in the F_1 and subsequent backcross progenies. Shetti et al. [17] also observed similar results with phi 057 SSR in maize. Similar results have been reported earlier by Bantte et al. [18] who noted clear and discrete polymorphism at *o2* locus with allele size of 165 bp in QPM donor parents and 159bp in non-QPM donor using marker *phi057*. Yang et al. [19], however, used successfully all the three gene targeted SSR markers namely *umc1066*, *phi057*, and *phi112* in detection of polymorphism at *o2* locus in 14 QPM and one non-QPM lines. Babu et al. [20] were the first in use of molecular markers in QPM breeding in India and successfully converted normal maize lines in QPM lines. They observed polymorphism between the normal and QPM donor inbred lines with all the three SSR markers however used only *phi057* in tracking of *o2* allele in backcross population since *phi112* exhibited dominant inheritance and therefore cannot be used in identification recessive *o2* allele in heterozygous condition from dominant *o2* allele in homozygous condition.

3.2 Detection of Heterozygotes at O2 Locus in BC_1F_1 Plants

The F_1 seeds from both the crosses were sown in five rows of 4.0 m length and populations of around 100 plants of F_1 were maintained. Two rows of normal maize lines of each combination were also sown alongside F_1 seeds. The F_1 population for each cross was screened for agronomic suitability considering phenotypic features such as overall morphology of plant vigour etc. The inferior plants from F_1s were also rogued out. The F1s were used as seed parent and normal maize lines were used as pollen parent and controlled hand pollinations were done to generate BC_1F_1 seeds on the F_1 plants in both (IML-187 X DQL-2029-1) and (BML-6 X DQL779-2-9). The crossing scheme is presented in Fig. 1. At maturity, ears on seed parents (BC_1F_1) were harvested dried and kept separately. The *opaque 2* gene is present in heterozygous (*O2o2*) condition in BC_1F_1 population and it will generate two types of gametes in seed parent i.e. *O2* and *o2* whereas in pollen population that is recurrent parent which is homozygous *O2O2* will produce only one type of gamete (*O2*). Thus, a backcross of a heterozygote with a homozygous recurrent parent would generate 50 percent heterozygous plants and 50 percent homozygous dominant plants at the given locus according to standard Mendelian segregation concepts. Thus in ideal and hypothetical conditions, union of two gametes from F_1s with one gamete from normal maize lines would produce equal number of plants with heterozygous and dominant alleles. However, in practical sense, the hypothetical ratio of 1:1 is seldom realized because observed ratio is dependent on probability theory. Further competitive ability of genes also distorts the ratio. In addition, section, sample size and sampling methods are other factors that influence the ratio of progenies in a population.

Chi square was therefore used to test the population of plants positive and negative for targeted locus. Ideally, the ratio should be 1:1 because the cross is a typical monohybrid test

cross where dominant and heterozygotes are expected in equal numbers. Chi square value of observed and expected frequencies of plants noted to be positive and negative marker phenotype ranged from 1.96 to 2.66. The populations exhibited non-significant chi square values therefore fit the expected ratio of 1:1. Here out of 96 plants used in genotyping from BC_1F_1 (A), 40 plants were heterozygous (*O2o2*) and 56 plants were homozygous (*O2O2*) resulting in a chi square ratio of 2.66 which is well below the tabulated value of χ^2 (3.841) at 1 degrees of freedom (Table 4). But after completion of flowering, phenotypic selection and 2nd backcrossing only 20 plants were retained and harvested because practically not all crosses are successful and not every plant survives to the harvesting stage. Similarly in BC_1F_1(B), out of 100 plants genotyped for O2 locus, 43 plants were found to be heterozygous (*O2o2*) and 57 plants were homozygous dominant (*O2O2*) resulting in a chi-square ratio of 1.96 which is well below the tabulated value of χ^2 (3.841) at 1 degrees of freedom. Therefore confirming the 1:1 backcross/test cross Mendelian ratio. The deviations from exact 1:1 ratio might be due to crossing over at *O2* locus [20]. As stated earlier by Babu et al. [20] identification of heterozygotes in the seedling stage prior to pollination aided in the rejection of non target BC progenies, resulting in substantial saving of labour and material resources. Magulama et al. [21] developed the two BC populations to employ marker assisted selection for *o2* gene. Gupta et al. [22] identified heterozygous (*O2o2*) progenies that occurred with 50% frequency in a given backcross population [23]. The heterozygotes identified in the progeny exhibited double band in the gel doc picture as presented in Fig. 3.

Table 1. Sequence of SSR primers

Marker	Sequence	Annealing T
Phi 057 :	F, 5'-CTCATCAGTGCCGTCGTCCAT-3' R, 5'-CAGTCGCAAGAAACCGTTGCC-3'	55^0C
Umc 1066	F, 5'-ATGGAGCACGTCATCTCAATGG-3' R, 5'-AGCAGCAGCAACGTCTATGACACT-3'	55^0C
phi 112	F, 5'-TGCCCTGCAGGTTCACATTGAGT-3' R, 5'-AGGAGTACGCTTGGATGCTCTTC-3'	55^OC

Table 2. Temperatures and time periods of cycles inPCR reaction

Stage	Temprature	Time	Cycle
1. Initial denaturation	94 °C	5 mins	1
2. a) Denaturation	94 °C	40 sec	
b) Annealing	50 °C-59 °C	40 sec	35
c) Extension	72 °C	1 min	
3. Final extension	72 °C	10 min	1
Hold at 4 °C			

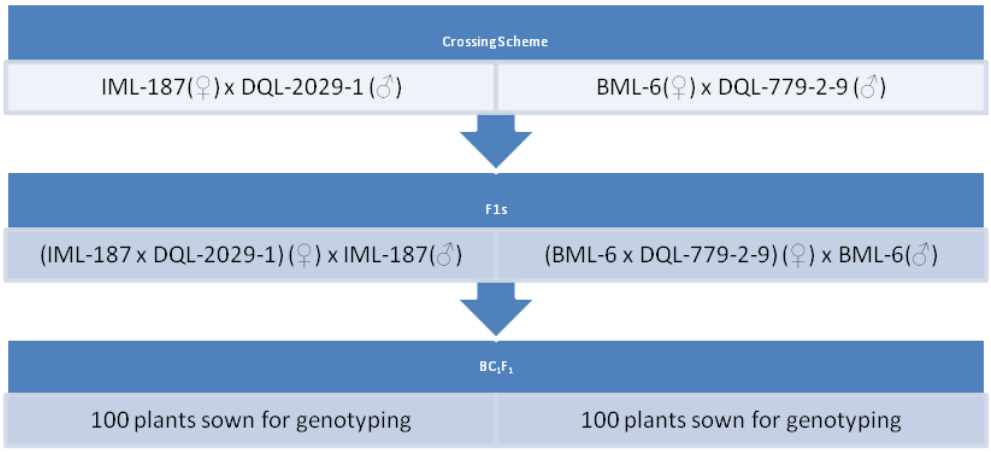

Fig. 1. Crossing scheme

Table 3. Polymorphism of markers to be used for foreground selection

S.No	Marker	IML-187	BML-6	DQL-2029-1	DQL-779-2-9	status
1	*Phi 112*	160bp	165bp	165bp	165bp	Inconclusive
2	*Phi 057*	155	160	170	170	Polymorphic
3	*Umc 1066*	160	165	170	160	Polymorphic

Table 4. Marker segregation at opaque locus at BC_1F_1 stage

S.No.	Crosses	BC_1F_1 Plants genotyped	Heterozygous plants	Homozygous plants	χ^2 ratio	χ^2 value (at 5% probability-3.841)
1	F1 x IML-187 BC_1F_1 (A)	96	40	56	2.66	Non significant
2	F1 x BML-6 BC_1F_1 (B)	100	43	57	1.96	Non significant

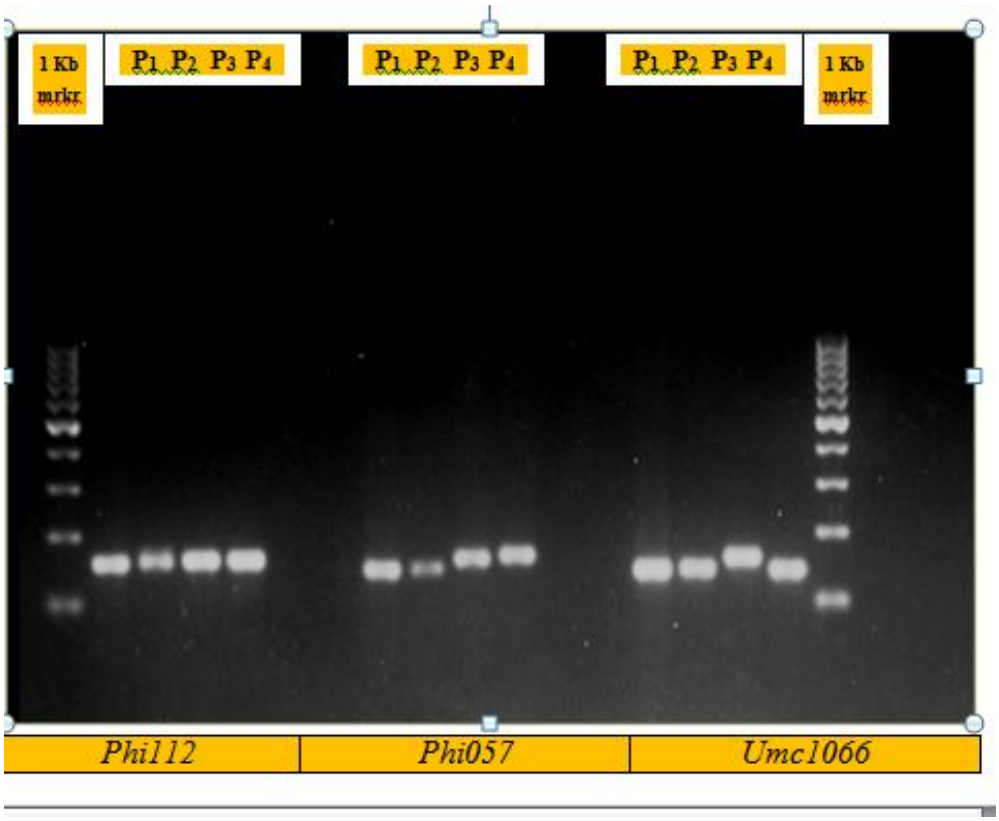

Fig. 2. Detection of polymorphic markers at *o2* locus in four parents viz. IML-187(P_1) 160 bpBML-6(P_2), DQL-2029-1(P_3) and DQL-779-2-9(P_4)

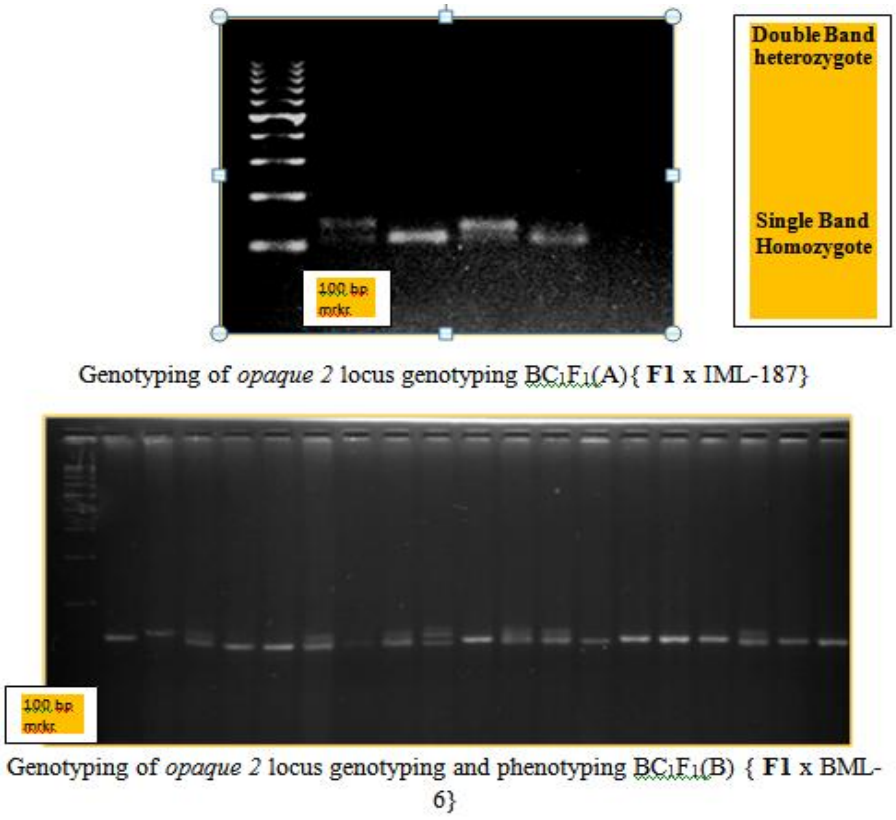

Fig. 3. Genotyping of *opaque 2* locus genotyping using *phi 057* SSR marker in BC_1F_1

4. CONCLUSION

Marker Assisted backcrossing is a useful tool in trait introgression as it helps in pre-flowering elimination of negative plants as well as donor genome and need for selfing is eliminated in BCF_1 s. SSR *phi-057* was able to clearly distinguish between QPM and normal parents and detect heterozygous plants in the backcross progeny. These selected heterozygous plants can be used for developing QPM version of SMH-5, by further backcrossing one more time. Background marker based selection is recommended before final attempt at recreating the QPM version of SMH-5.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Coleman CE, Larkins BA. The prolamins of maize. In: Seed proteins. Dordrecht: Springe; 1999;109-39.
2. Tomé D, Bos C. Lysine requirement through the human life cycle. J Nutr. 2007; 137(6);Suppl 2:1642S-5S. DOI:10.1093/jn/137.6.1642S,PMID:17513440
3. Osgood-Zimmerman A, Millear AI, Stubbs RW, Shields C, Pickering BV, Earl L et al. Mapping child growth failure in Africa between 2000 and 2015. Nature. 2018;555(7694):41-7. DOI: 10.1038/nature25760, PMID:29493591
4. Gibbon BC, Larkins BA. Molecular genetic approaches to developing quality protein maize. Trends Genet. 2005;21(4):227-33. DOI: 10.1016/j.tig.2005.02.009, PMID:15797618
5. Motukuri SK. Quality protein maize: an alternative food to mitigate protein deficiency in developing countries. In: Maize-production and use. Intech Open; 2019.
6. Vasal SK. The quality protein maize story. Food Nutr Bull. 2000;21(4):445-50. DOI: 10.1177/156482650002100420
7. Vasal SK, Villegas E, Bjarnason M, Gelaw B, Goertz P. Genetic modifiers and breeding strategies in developing hard endosperm opaque-2 materials. In: Pollmer WG, Phillips RH, editors. Improvement of quality traits of maize for

grain and silage use. London: Martinus-Nijhoff. 1980;3773.
8. Villegas E, Vasal SK, Bjarnason M. Quality protein maize – what is it and how was it developed. In: Mertz ET, editor. Quality protein maize. St Paul, MN: American Society Cereal Chemistry. 1992;27-48.
9. Geevers HO, Lake JK. Development of modified opaque-2 maize in South Africa. In: Mertz ET, editor. Quality protein maize. St Paul, MN: American Society Cereal Chemistry; 1992. p. 49-78.
10. Krivanek AF, De Groote H, Gunaratna NS, Diallo AO, Friesen D. Breeding and disseminating quality protein maize (QPM) for Africa. Afr J Biotechnol. 2007;6:312-24.
11. Vivek BS, Krivanek AF, Palacios-Rojas N, Twumasi-Afriyie S, Diallo A. Breeding quality protein maize (QPM): protocols for developing QPM cultivars. Mexico: CIMMYT, D.F. 2008;105.
12. Vasal SK, Srinivasan G, Pandey S, González FC, Crossa J, Beck DL. Heterosis and combining ability of CIMMYT's quality protein maize germplasm: I. lowland tropical. Crop Sci. 1993;33(1):46-51. DOI:10.2135/cropsci1993.0011183X00330 0010006x
13. Bänziger M, Diallo AO. Progress in developing drought and N stress tolerant maize cultivars for eastern and southern Africa. In: Friesen DK, Palmer AFE, editors. Integrated approaches to higher maize productivity in the new millennium. Proceedings of the 7th eastern and southern Africa regional maize conference. Nairobi, Kenya: CIMMYT/ KARI. 2004;189-93.
14. Bänziger M, Edmeades GO, Beck D, Bellon M. Breeding for drought and nitrogen stress tolerance in maize: from theory to practice. México, DF: CIMMYT; 2000.
15. Yadav OP, Hossain F, Karjagi CG, Kumar B, Zaidi PH, Jat SL. Genetic improvement of maize in India: retrospect and prospects. Agric Biol Res. 2015;4: 325-38.
16. Prasanna BM, Pixley KV, Warburton ML, Xie C. Molecular markerassisted breeding for maize improvement in Asia. Mol Breeding. 2010;26(2):339-56. DOI: 10.1007/s11032-009-9387-3
17. Shetti P, Sagare DB, Surender M, Reddy SS. Development of lysine and tryptophan rich maize (Zea mays) inbreds employing marker assisted backcross breeding. Plant Genet. 2020;23:100236. DOI: 10.1016/j.plgene.2020.100236
18. Bantte K, Prasanna BM. Simple sequence repeat polymorphism in quality protein maize (QPM) lines. Euphytica. 2003; 129(3):337-44. DOI: 10.1023/A:1022257021205
19. Yang W, Zheng Y, Ni S, Wu J. Recessive allelic variations of three microsatellite site within the *o2* gene in maize. Plant Mol Biol Rep. 2004;22(4):361-74. DOI: 10.1007/BF02772679
20. Babu R, Nair SK, Kumar A, Venkatesh S, Sekhar JC, Singh NN et al. Two generation marker aided backcrossing for rapid conversion of normal maize lines to quality protein maize (QPM). Theor Appl Genet. 2005;111(5):888-97. DOI: 10.1007/s00122-005-0011-6, PMID 16034586
21. Magulama EE, Sales EK. Marker-assisted introgression of *opaque* 2 gene into elite maize inbred lines. USM R D. 2009;17(2):131-5.
22. Gupta HS, Raman B, Agrawal PK, Mahajan V, Hossain F, Thirunavukkarasu N. Accelerated development of quality protein maize hybrid through marker-assisted introgression of *opaque-2* allele. Plant Breed. 2013;132(1):77-82. DOI: 10.1111/pbr.12009
23. Vivek BS, Krivanek AF, Palacios-Rojas N, Twumasi-Afriyie S, Diallo AO. Breeding quality protein maize (QPM): Protocols for developing QPM cultivars. México, DF: CIMMYT; 2008.