Received: 14 Oct 2021 | Revised: 18 Jun 2022 | Accepted: 20 Jun 2022

Electronic Journal of Plant Breeding

Research Article

Development of tryptophan rich maize lines through simple sequence repeat marker aided introgression of *opaque2* trait

Swaran Lata[1], Reena Kumari*[2], Kalpna Thakur[1], Maneet Rana[3], Naresh Thakur[2] and Waseem Hussain[4]

[1]Department of Crop Improvement, College of Agriculture,CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur, Himachal Pradesh, India, 176062,
[2]Department of Biotechnology, College of Horticulture and Forestry, Neri, Hamirpur (HP), 177001,
[3]Department of Crop Improvement, ICAR-Indian Grassland and Fodder Research Institute, Jhansi (UP), 284 003,
[4]Department of Agronomy and Horticulture, University of Nebraska, Lincoln- 68583-0915, USA
***E-Mail:** reenarattan@gmail.com

Abstract
Maize is the main staple food of the world but it is nutritional deficient due to the scarcity of two essential amino acids viz., lysine and tryptophan. Under this study, *opaque2* gene from QPM donor (CML-169) was transferred into normal maize (BAJIM-08-26) through marker aided backcross breeding method. The *opaque2* specific SSR marker (*phi057*) was used for foreground selection in BC_1F_1 and BC_2F_1 generations. The heterozygous offspring were self-pollinated to produce the BC_2F_2 generation. Further plants on the basis of 25% opaqueness were selected and selfed to develop BC_2F_3 and BC_2F_4 progenies. The background selection using a series of SSR markers showed 97% recovery in the recurrent parent genome of backcrossed generation. Tryptophan content and total protein in the endosperm of BC_2F_4 progenies were found to be ranged from 0.75 to 0.93% and 7.0 to 9.6 %, respectively. As an outcome, three promising MAS derived QPM lines viz., B76-22-2, B70-5-8 and B42-3-1 were developed that showed a high percentage of tryptophan, grain yield and recovery of recurrent parent genome. The worldwide problem of protein malnutrition could be improved by adopting a molecular breeding approach for the development of nutritionally rich maize over the normal maize.

Keywords: Marker assisted backcrossing, *opaque2*, tryptophan content, SSR markers, QPM lines and normal Maize

INTRODUCTION

Maize is one of the most important food crops in developing countries. In India, maize is considered an important cereal crop as it is utilised as a source of feed and food, it stands at third position as a staple food after wheat and rice (Yadav *et al*., 2015). Maize endosperm has 1.5– 2.0% lysine and 0.25–0.50% tryptophan which classifies it as a nutritionally poor crop. The optimal human nutrition requirement for lysine and tryptophan is 5.0% and 1.1%, respectively. There are number of studies available on mutations in maize which cause a decrease in zein content followed by an increase in essential amino acids and storage protein content (Mertz *et al*., 1964; Nelson *et al*., 1965; Misra *et al*., 1972; Tsai and Dalby, 1974). These mutations includes *opaque* 2 (*o2*), *opaque* 7 (o7), *brittle*-1 (bt1), *brittle*-2 (bt2), *shrunken*-1 (sh1), *shrunken*-2 (sh2), *shrunken*-4 (sh4), *floury*-2 (fl2), *sugary*-1 (su1), etc. Introgression of any of above mentioned mutants in the normal maize could be used to increase the biological value of traditional maize. Among all these mutants *o2* is most widely used in maize

breeding programs to improve the lysine and tryptophan content of normal maize endosperm. This gene was reported to have pleiotropic effects and responsible to affect other agronomic importance traits also. The *o2* maize kernel appeared to be chalky and dull and had soft endosperm. It is also susceptible to pests and resulted in low grain yield (Krivanek *et al*., 2007). Various methods have been searched to improve the characteristics of *o*2 maize. Normally o2 gene was found to enhance the lysine content by lowering the level of zein protein. However, *opaque* 2 modifiers were reported to alter the soft endosperm into hard. Therefore, breeders use the *o*2 gene in combination with endosperm and amino acid modifier genes to develop quality protein maize having more lysine and tryptophan content as compared to normal maize (Larkins *et al*., 2017). Quality protein maize (QPM) with hard endosperm was reported and named by International Maize and Wheat Improvement Center (CIMMYT, Mexico). In view to solve the problem of malnutrition around the world the promotion of quality protein maize (QPM) varieties globally is considered to be a good approach (Nyakurwa *et al*., 2017).

With the advancement in genomic and molecular research, it has become convenient to select promising individuals with desirable traits and development of new varieties and hybrids in a short duration through molecular breeding (Tripathy *et al*., 2017; Lekhi *et al*., 2018 and Pukalenthy *et al*., 2019). The effectiveness of molecular breeding could be authenticated through foreground and background selection. The foreground selection is done to screen the individuals having desirable trait of interest introgressed from donor parent and the recovery of recurrent parent genome in the individuals were analysed through background selection (Babu *et al*., 2004). Molecular markers associated with the *o*2 phenotype and *o*2 modifiers have been identified for a successful QPM breeding programme (Babu *et al*., 2015). The *opaque 2* homozygous (*oo*) and heterozygous (*Oo*) individuals have been identified using gene specific simple sequence repeat (SSR) markers *viz*., *phi057*, *phi112* and *umc1066* (Babu *et al*., 2005; Gupta *et al*., 2009; Kostadinovic *et al*., 2015; Surender *et al*., 2017). The marker assisted back cross introgressed *o*2 maize inbred lines were found to have variation in the endosperm textures (Ren *et al*., 2018).

There is a constant need to develop high yielding maize lines with enhanced nutrition quality. This study was, therefore, undertaken to develop medium-maturity QPM inbreds with high tryptophan content.

MATERIALS AND METHODS

In this study, initially six normal maize (HKI-163, HK1-193, BAJIM-08-26, BAJIM-08-96, BAJIM-08-34 and BAJIM-08-27) of Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishwavidalaya, Palampur, Himachal Pradesh and seven QPM inbred lines (CML-141, CML-168, CML-169, CML-193, CML-190, CML-173 and CML-189) developed by International Center for Maize and Wheat Research (CIMMYT), Mexico were used and screened for polymorphism using gene specific SSR marker *phi057* associated with *o*2 gene. The sequence of *phi057* forward primer was 5'CTCATCAGTGCCGTCGTCCAT 3' and reverse primer was 5'CAGTCGCAAGAAACCGTTGCC3'.

After polymorphism screening, one normal maize inbred BAJIM-08-26 was selected as recipient parent and CML-169 as *opaque*2 donor parent. F_1 (BAJIM-08-26 x CML-169) was generated and backcrossed to the recipient parent BAJIM-08-26 to get BC_1F_1. DNA was extracted from backcross generations and foreground selection was done using a *phi057* SSR primer in order to identify heterozygous plants. In total 47 heterozygotes were identified and backcrossed with the recipient parent to get BC_2F_1 generation. The BC_2F_1 generation was planted and selected heterozygotes were selfed to get BC_2F_2 for validation of phenotyping results. Foreground selection and background selection were done in both BC_2F_1 and BC_2F_2 generations for the identification of heterozygotes and recurrent parent genome, respectively. In background selection, 120 SSR markers were screened among the progenies of BC_2F_1 and BC_2F_2, respectively (**Table 1**). All the selected plants of BC_2F_2 were selfed and their kernels were screened for the modification under transmitted light using a light box. The kernels were grouped under five classes of modification viz., 0, 25, 50, 75 and 100% opaque.

Genomic DNA was extracted from the young leaf tissue of maize inbreds using cetyl tri-methyl ammonium bromide (CTAB) method (Murray and Thompson, 1980 and Thakur *et al*., 2015) with slight modification in the protocol. Isolation was done from fresh leaves by grinding in liquid nitrogen and suspending the powder in an extraction buffer containing Tris HCl, NaCl, CTAB and β-mercaptoethanol. This was followed by suspension in chloroform: isoamyl alcohol (24:1). DNA was precipitated by prechilled isopropanol or ethanol. Precipitated DNA was dissolved in TE buffer. Then DNA was purified, quantified and stored at -20ºC for further use or used directly for PCR amplification reactions. PCR was carried out in a 96 wells BIORAD PCR system. The PCR profile consisted of initial denaturation at 94ºC for 5 min., Final denaturation at 94ºC for 1min, annealing at 55-65ºC for 1 min, extension at 72ºC for 2 min and final extension at 72ºC for 10 minutes. The amplified product was separated on 3.5% agarose gel (Himedia). SSR polymorphism at *opaque*2 locus was assessed by PCR amplification of genomic DNA with *phi057* primer. Around 120 SSR markers spanning all the bin locations in a maize SSR consensus map were used in background selection to screen the polymorphic markers between recurrent and donor parents. The SSR markers which were found to be polymorphic among parents were further used in background selection to determine per cent recurrent parent genome recovery at each backcross generation.

Table 1. List of SSR primers used for background selection

S.No.	Primers	Sequence (Forward and Reverse primer)	S.No.	Primers	Sequence (Forward and Reverse primer)
1.	umc 1353	AGACAGGATCATCGAAAACACACA ACCTCAGCCTCCTCGTCAACTACT	24.	bnlg 1600	CGATCAGTGCGTGGAGAGTA TAGGCATGCATTGTCCATTG
2.	bnlg 1083	ACAGTCTGTTGGGGAACAGG CAACGCTGGTTTGTCGTTTA	25.	umc 1178	CTGTCGTAAGAGCGCCAACAG GTCTGAACGATGAACAGTACACGC
3.	umc 2240	CGCCTTTGTAACCCAGACTCATTA CGGATGTTGCCAAGTACATCATATC	26.	umc 1083	CTTTCCTCTCTGGAGCGTGTATTG ATATGTTGCAGAACCATCCAGGTC
4.	bnlg 1347	GTGGTCACGACGAAATCCTT TTGCAATCACAGGTGGTT	27.	phi 057	CTCATCAGTGCCGTCGTCCAT CAGTCGCAAGAAACCGTTGCC
5.	phi 083	CAAACATCAGCCAGAGACAAGGAC ATTCATCGACGCGTCACAGTCTACT	28.	umc 1456	GCCACAGCTCACTAGCTCAAA CTCTGTGTGTTTGCTTGATTG
6.	umc 2258	GAATAAGACCAGACAGCACCG AAGATTGTATAAATGGCAGCC	29.	umc 1710	ACTTTGCAACTACCGTACATGGGT TTCGACTGCACGTGAAAATCTATC
7.	bnlg 420	CTTGCGCTCTCCTCCCCTT GGCCAGCTCACTGCTCACT	30.	bnlg 1017	ATTGGAAGGATCTGCGTGAC CAGCTGGTGGACTGCATCTA
8.	umc 2265	AAGACGGTCCCGAAGAAAGC CTGGACGTGGACTCAGACACC	31.	bnlg 1832	GCGCCCACAACAAGTAAATT CCTCATTGTAAGGGGCAGAA
9.	bnlg 1755	CCTAGTAGACCTCACCGCCA GGAGTTCACCGATGGCAC	32.	bnlg 1523	GAGCACAGCTAGGCAAAAGG CTCGCACGCTCTCTCTTCTT
10.	umc 2139	ATAAGGAACATCCCACCTGTTTT GGTGTGCTGGGTTCTTGTGG	33.	bnlg 1917	ACCGGAACAGACGAGCTCTA TTTGCTTCCAACTCACATGC
11.	umc 2332	GTCGGAGAAGGAGCTACTGAGCTA CACAGGTACGTCTGGATGCTGT	34.	bnlg 589	ACCGGAACAGACGAGCTCTA GCGACAGACAGACAGACAAGCGCATTGT
12.	phi 115	GCTCCGTGTTTCGCCTGAA ACCATCACCTGAATCCATCACA	35.	umc 2063	GGACTGAAGCGTGGAATGTTCT ATCGCAATCTGAGACCACTTGTT
13.	umc 1872	CTTTTGTGATGTCTGCAATATGCC TTAGTAGGTGCATTGGATGCTCAA	36.	umc 1859	ATATACATGTGAGCTGGTTGCCCT GCATGCTATTACCAATCTCCAGGT
14.	umc 2358	GCACGAGGTTTCCCTTGCTC GACTCGCGAATAAGGTCTGGG	37.	umc 1592	GACCATATGTGCTCCAAAACCTTC AAGCTTCTTCGGTCTTTGTAGGGT
15.	umc 2371	GATTATTTGATTAGCCCGGTTGGT CTGAGTCGTATTTATAGAGCCCGC	38.	umc 1061	AGCAGGAGTACCCATGAAAGTCC TATCACAGCACGAAGCGATAGATG
16.	umc 2017	AGAGGTTACTACGGAGTGTGGCAG GTCAGGGTACTGCTTCTCGAACTC	39.	umc 1555	ATAAAACGAACGACTCTCTCACCG ATATGTCTGACGAGCTTCGACACC
17.	umc 2043	GAGGCATACGGCATACATAC GTAGGAGAAACAGGTGCTGGT	40.	umc 2331	CGGTGAGTCAGTGAGTGAGTC AAGAACTGCAAAAAGGTACCC
18.	umc 1128	TCAATTTTGAGCTATCACTTTCCG ATTGGTTCCATTGGTTTTGTTGAT	41.	bnlg 2077	GACCAGAGGATGGGGAAATT GTAGGCACATGCACATGAGG
19.	phi 064	CCGAATTGAAATAGCTGCGAGAACCT ACAATGAACGGTGGTTATCAACACGC	42.	umc 1970	ACTGATGGTGTTCTTGGGTGTTTT TTTTTACCCGAAGGTTCATCGTTT
20.	phi 079	TGGTGCTCGTTGCCAAATCTACGA GCAGTGGTGGTTTCGAACAGACAA	43.	bnlg 490	GCCCTAGCTTGCTAATTAACTAACA ACTGTAAGGGCAGTGGACCTATA
21.	umc 2200	CTTCCTCCTCCTCGTCTCTTTCAT GGGGCCAAATCTGAATCTTCC	44.	phi 076	TTCTTCCGCGGCTTCAATTTGACC GCATCAGGACCCGCAGAGTC
22.	umc 1056	CGGATCGCTTTTTACCGTCTA AGCAAGAGTACGCTTCCATTT	45.	umc 2373	ACCCAAGTGAGGTGAAGTGAAGC TATGGTACAGGCACAGCAGCAAGTA
23.	umc 2298	ATCCACTCCCAAGTCCCAACAC CTTCTTCCGGTTCTTCTTCAGGC	46.	umc 2201	AGGGAAGGGGAAAAGCAGTTAAG TAGAACGGCGAACAGAAGCAG

47.	umc 1178	CTGTCGTAAGAGCGCCAACAG GTCTGAACGATGAACAGTACACGC
48.	umc 2325	CCTAGGAACTCTGATGGCTATGGA CTACGATATCCACCTCTACCACCG
49.	phi 065	AGGGACAAATACGTGGAGACACAG CGATCTGCACAAAGTGGAGTAGTC
50.	umc 1044	CACCAACGCCAATTAGCATCC GTGGGCGTGTTCTCCTACTACTCA
51.	bnlg 1811	ACACAAGCCGACCAAAAAAC GTAGTAGGAACGGGCGATGA
52.	phi 059	AAGCTAATTAAGGCCGGTCATCCC TCCGTGTACTCGGCGGACTC
53.	phi 031	GCAACAGGTTACATGAGCTGACGA CCAGCGTGCTGTTCCAGTAGTT
54.	umc 1333	AGGTAAGCGAGCATCTGAGGGT TCTGGAGACTCTTCTGGGTGAACT
55.	umc 1282	TACACTACACGACTCCCAACAGGA GCGAGGGTTCTTTCCATAGAGAAT
56.	umc 2230	AACGCGACGACTTCCACAAG ACACGTAATGTCCCTACGGTCG
57.	bnlg 1273	AAACACCAAACGTCACGTGG GGCGACGAGATACAGGATGT
58.	umc 1013	TAATGTGTCCATACGGTGGTGG AGCTGGCTAGTCTCAGGCACTC
59.	bnlg 1839	AGCAGACGGAGGAAACAAGA TCTCCCTCTCCCTCTTGACA
60.	bnlg 1520	TCCTCTTGCTCTCCATGTCC ACAGCTGCGTAGCTTCTTCC
61.	umc 1506	AAAAGAAACATGTTCAGTCGAGCG ATAAAGGTTGGCAAAACGTAGCCT
62..	umc 2047	GACAGACATTCCTCGCTACCTGATCT GCTAGCTACCAAACATTCCGAT
63.	umc 1122	CACAACTCCATCAGAGGACAGAGA CTGCTACGACATACGCCA GGC
64.	umc 1136	CTCTCGTCTCATCACCTTTCCCT CTGCATACAGACATCCAACCAAAG
65.	umc 1152	CCGAAGATAACCAAACAATAATAGTAGG ACTGTACGCCTCCCCTTCTC
66.	umc 1399	GCTCTATGTTATTCTTCAATCGGGC GGTCGGTCGGTACTCTGCTCTA
67.	umc 1555	ATAAAACGAACGACTCTCTCACCG ATATGTCTGACGAGCTTCGACACC
68.	umc 1225	CTAGCTCCGTGTGAGTGAGTGAGT TTCCTTCTTTCTTTCCTGTGCAAC
69.	umc1335	ATGGCATGCATGTGTTTGTTTTAC ACAGACGTCGCTAATTCCTGAAAG
70.	umc 1424	CCGGCTGCAGGGGTAGTAGTAG ATGGTCAGGGGCTACGAGGAG
71.	umc 1165	TATCTTCAGACCCAAACATCGTCC GTCGATTGATTTCCCGATGTTAAA
72.	umc 1403	GTACAACGGAGGCATTCTCAAGTT TGTACATGGTGGTCTTGTTGAGGT
73.	phi 127	ATATGCATTGCCTGGAACTGGAAGGA AATTCAAACACGCCTCCCGAGTGT
74.	phi 056	ACTTGCTTGCCTGCCGTTAC CGCACACCACTTCCCAGAA
75.	bnlg 1258	GGTGAGATCGTCAGGGAAAA GAGAAGGAACCTGATGCTGC
76.	bnlg 1194	GCGTTATTAAGGCAAGCTGC ACGTGAAGCAGAGGATCCAT
77.	umc 1827	GCAAGTCAGGGAGTCCAAGAGAG CCACCTCACAGGTGTTCTACGAC
78.	umc 1324	ATCCATCATCATCATCATTGCTTG ATGTCATCATGTACCAGGTGTTGG
79.	umc 1782	CGTCAACTACCTGGCGAAGAA TCGCATACCATGATCACTAGCTTC
80.	umc 2355	CTACTCCCCGAAGCCGTCTAAG CGGGTTGTTGTTGGAGTAGGAC
81.	bnlg 1782	CGATGCTCCGCTAGGAATAG TGTGTTGGAAATTGACCCAA
82.	phi 080	CACCCGATGCAACTTGCGTAGA TCGTCACGTTCCACGACATCAC
83.	umc 1040	CATTCACTCTCTTGCCAACTTGA AGTAAGAGTGGGATATTCTGGGAGTT
84.	umc 1006	AATCGCTTACTTGTAACCCACTTG AGTTTCCGAGCTGCTTTCTCT
85.	umc 1887	CTTGCCATTTTAATTTGGACGTTT CGAAGTTGCCCAAATAGCTACAGT
86.	umc 1695	CAGGTAATAACGACGCAGCAGAA GTCCTAGGTTACATGCGTTGCTCT
87.	umc 1265	GCCTAGTCGCCTACCCTACCAAT TGTGTTCTTGATTGGGTGAGACAT
88.	umc 2129	ACGTGGTCATCACTCACCGC AAGGAGGAGCGTTCTCGTGG
89.	umc 1257	CAACGGAAGTGGCTGTAGAGTTTT ACAGAGCATGTCAGGTATTTGCAG
90.	bnlg 1784	GCAACGATCTGTCAGACGAA TTGGCATTGGTAATGGGTCT
91.	umc 1418	TCACACACACACTACACTCGCAAT GAGCCAAGAGCCAGAGCAAAG
92.	bnlg 557	TCACGGGCGTAGAGAGAGA CGAAGAAACAGCAGGAGATGAC
93.	bnlg 1046	TGAGCCGAAGCTAACCTCTC GATGCAAAGGAGGTTCAGGA
94.	phi 087	GAGAGGAGGTGTTGTTTGACACAC ACAACCGGACAAGTCAGCAGATTG

95.	umc 1492	GAGACCCAACCAAAACTAATAATCTCTT CTGCTGCAGACCATTTGAAATAAC	108.	umc 1227	CAAGTTGGTGAGATGGATCTGTTG GCTCCTGGGTCTTCCTCTCC
96.	umc 1310	GAGGAAGAGTTGGCCAGGATG AACTCCGAGATCTACGACAACAGC	109.	umc 2391	ACCAGGAGAAGAAGAACCAGCA GTGTCCCTCCTCCTTGTGGTC
97.	umc 2375	GCCGTACTGATGTGATGGTCC TCTGACATTGTCCTCTTGACCAAA	110.	umc 1363	TGTTTAAGTGTTGGCAGAAAGCAA TCTCCCTCCCCTGTACATGAATTA
98.	umc 1256	TCGAGTTTGCTTCTCTCCAGTTTC TGCAGCATATGGCTCTTTATTCAA	111.	umc 1109	GCAACACAGGACCAAATCATCTCT GTTCGGTCCGTAGAAGAACTCTCA
99.	umc 1369	TTCCAGCACTAACTTACAGCAACG AGATATGCGTATGGCTCTTGTTGG	112.	umc 1285	AAACTGGATATGGTTGGTTGGTTG TAAATATACGGCCCCAAGAAAACC
100.	umc 1505	TTACACAGAAGCCCATTTGAAGGT GGATGGTTGTTGGTGGTGTAGAAT	113.	umc 1483	GTTAGGGGGTAGAAGACAGGGATG GTTCAAGGCCATTGTAATCCTCCT
101.	umc 1175	ACACCCCAAAACTCACTTAATCCA CCCTCGTAGTCTGTCAAGGTTTTG	114.	umc 2212	CATGGATCCACTGTTTCTTTGCTA ATGCCAATCCTAAAGGGCGT
102.	umc 1381	CTCTAGCTACGAGCCTACGAGCA CCGTCGAGTCAACTAGAGAAAGGA	115.	umc 1751	CCTCATAAAACCAGCAGATCCCT GCTTTTGTGTATACTGGTTTCGCC
103.	umc 2220	AATACAAGAAGCGAAAGGGGAAAG GCGTAGCAAACCAAAGAAGAAGAA	116.	umc 1538	AGAAACAACACATTCCCTCGAAAC AGCAGCTTTTACCCCTGATTTTTC
104.	bnlg 1456	TTCATGAGGACCGTGTTGAA CTCTAGGTGGTTAAGATTAACTCATT	117.	umc 1829	GTTGATTGGTTGATGTGGAAACAA CAGTTTGATGTTCATGGCTCTCTC
105.	umc 1800	TTATGGGTGCTGGTGATGTGTATC GAAAAGCAATCGCTTCTGAGAAAA	118.	umc 1521	GAGTCAGCTTCACCTCTCGATCTC GTCTCTCTCTTGCATGCCACTAGC
106.	umc 1704	TTCACCGGGTAGTCCTTCTTACTG AAGTACGCTGTACGCAGGCAG	119.	umc 1591	GAGGTCTCTCTCGGTCGACATC CAACCAACTGGCAACTACTCGAC
107.	umc 2142	ATGGATCAGGGGAAAGAGCAA CCTCCTCGTCCTCCTTCTTGAT	120.	umc 1792	CATGGGACAGCAAGAGACACAG ACCTTCATCACCTGCAACTACGAC

In maize kernel opacity is directly correlated with a hardness of endosperm. Maize endosperm varies from vitreous (hard) to opaque (soft) endosperm representing the variation in kernel phenotype (Pomeranz *et al*., 1984). In this study, the opacity of kernel indicated the hardness of endosperm. The evaluation of kernels opacity was carried out by using light box (Vivek *et al*., 2008). Kernel modification was assessed and grains were grouped as hard, semi-soft and soft. The kernels with less than 25% opaqueness were selected to develop BC_2F_3 or advanced generations. Selected plants of BC_2F_3 generation were selfed to get BC_2F_4 generation The per cent tryptophan content in seeds of BC_2F_4 generation was estimated.

Twenty-five seeds from BC_2F_4 plants were soaked in distilled water for 25 min. before removing the pericarps and embryos. The endosperms were air dried overnight and were ground (to approximately 0.1 mm) in a cyclone mill (Retsch, ZM 1000) followed by deffating with 100% hexane in a Soxhlet-type continuous extractor (Buchi, B-811). The defatted samples were analyzed for tryptophan content using a spectrophotometer (Spectronic, Genesys 2) as described by Mertz *et al*. (1975). Micro Kjeldahl' method (Kjeldahl, 1883) was used to determine the total protein content in seeds of BC_2F_4 plants. All the biochemical analyses were performed in triplicates.

Twelve selected BC_2F_4 lines of the cross BAJIM-08-26 X CML 169 were evaluated for different agronomic traits like plant height, cob placement height, days to 75% maturity, days to 50% pollen shed, days to 50% silking, grain yield along with their parents at the experimental field of Department of Crop Improvement, Chaudhary Swaran Kumar Himachal Pradesh Krishi Vishwavidalaya, Palampur and Regional Research Station, Bajaura. Complete Randomised Block Design with two replications was used to evaluate the data. The plants were raised in row of 3m length with a plant to plant distance of 20 cm and row to row distance of 60 cm.

RESULTS AND DISCUSSION

Parental polymorphic analysis of six normal and six quality protein maize inbred lines was conducted using SSR marker (*phi 057)* associated with *opaque2* gene. Polymorphism was reported between QPM donor, showing a band at around 150 bp and the non-QPM line showing a band at around 140 bp with *phi 057* (**Fig. 1**). Marker assisted selection is a shortcut to achieve the successful transfer of important traits in the desirable crop, which could be otherwise expensive, time consuming and laborious through conventional breeding. The present investigation was carried out to develop the modified maize having a high content of tryptophan than

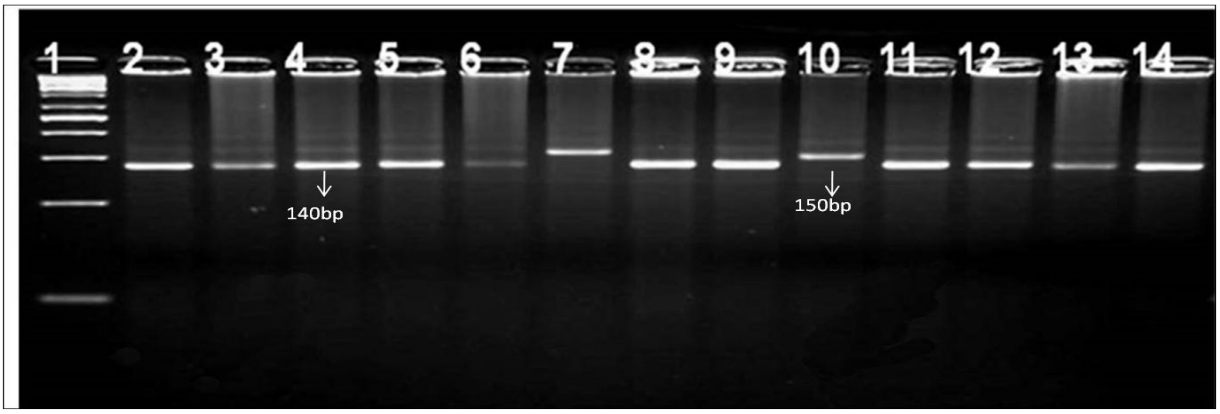

Fig. 1. Parental polymorphism using *opaque 2* specific SSR marker *phi 057* Lanes: 1=50 bp ladder, 2 =HKI-163, 3=HKI-193, 4=BAJIM-08-26, 5=BAJIM-08-96, 6= BAJIM-08-34, 7=BAJIM-08-27, 8= CM-141, 9= CML-168, 10=CML-169, 11=CML-193, 12=CML-170,13=CML-173,14=CML-189

normal maize varieties. This could be achieved through introgression of the *o2* recessive allele from QPM donor to normal maize.

In this study *opaque*2 gene specific SSR marker *phi 057* was used to select polymorphic parents. Babu *et al*. (2005) and Danson *et al*. (2006) reported the polymorphism between QPM and normal inbreds using *phi057* marker. Similarly, there are number of reports available in which the opaque 2 loci were found to be polymorphic between QPM (donor parent) and normal (recurrent parent) maize (Gupta *et al*., 2013; Hossain *et al*., 2018; Zunjare *et al*., 2018).

Foreground selection for *opaque*2 gene in BC_1F_1 generation was carried out using *phi057* marker for identifying heterozygous progenies. In BC_1F_1 population of BAJIM-08-26× CML-169, 112 plants were found to be heterozygous for *opaque*2 gene out of a total of 250 plants (**Fig. 2**). Only *opaque*2 gene specific SSR marker identified heterozygotes were further selected and backcrossed to develop BC_2F_1 generation. The co-dominant nature of polymorphism exhibited by this marker successfully differentiates between homozygote's and heterozygote's backcross progeny.

In BC_2F_1 population of BAJIM-08-26× CML-169, 90 plants out of 188 were found to be heterozygous for *opaque*2 gene. The chi-square test for goodness of fit showed that the marker was segregated according to the expected Mendelian ratio of 1:1 for a BC_1 and BC_2F_1. Babu *et al*. (2005) also noted similar observations and used *opaque*2 gene specific SSR markers *umc1066* in tracking of *o2* allele in backcross population. Magulama *et al*. (2009) noted polymorphism at *o2* locus with *phi057* and *umc1066*, however, they applied only *phi057* in marker-assisted selection for the development of backcross populations. Because of the reliability and discrete polymorphism, *phi057* SSR marker has also been used earlier in marker-assisted. In backcross generations (Manna *et al*., 2005; Danson *et al*., 2006; Jompuk *et al*., 2011) observed allele sizes of 160bp and 170bp in *o2* and normal maize lines, respectively when *o2* locus was genotyped using marker *phi057*. Gupta *et al*. (2013) used successfully *umc1066* marker in foreground selection to identify plants heterozygous at *o2* locus in backcross generations and also to select the homozygotes in selfed generation for QPM hybrid development.

In this study, background selection for recurrent parent genome was done in both BC_2F_1 and BC_2F_2 generations. Out of 120 SSR markers, only 80 markers were found to show polymorphism among recurrent and donor parents. Further background selection in BC_2F_1 was carried out using polymorphic markers. The recovery of recurrent parent genome in this backcrossed generation was found to be ranged between 83.04 to 91.07%. In BC_2F_2 generation 102 plants were genotyped, out of which 40 were reported to be recessive homozygotes. Background selection of this generation progeny was performed with only 69 polymorphic markers. The average recurrent parent genome content of BC_2F_2 generation was 87.70%. Out of 40 plants, B-70-4 and B-76-22 had the highest proportion of recurrent parental genome of 91.66 and 93.33%, respectively. In BC_2F_3 generation, the seeds of *o2* homozygous plants were carried forward for light box test through marker assisted selection in BAJIM-08-26×CML-169. The objective of the background selection is to recover the maximum proportion of recurrent parent genome at non-target loci through markers that are distributed evenly throughout the genome (Hospital *et al*., 1992). In this study, background selection for recurrent parent genome was done in both BC_2F_1 and BC_2F_2 generation with 86 and 69 primers with an average recovery of recurrent parent genome were found to be 83.04 % and 87.7%. The number of studies reported that

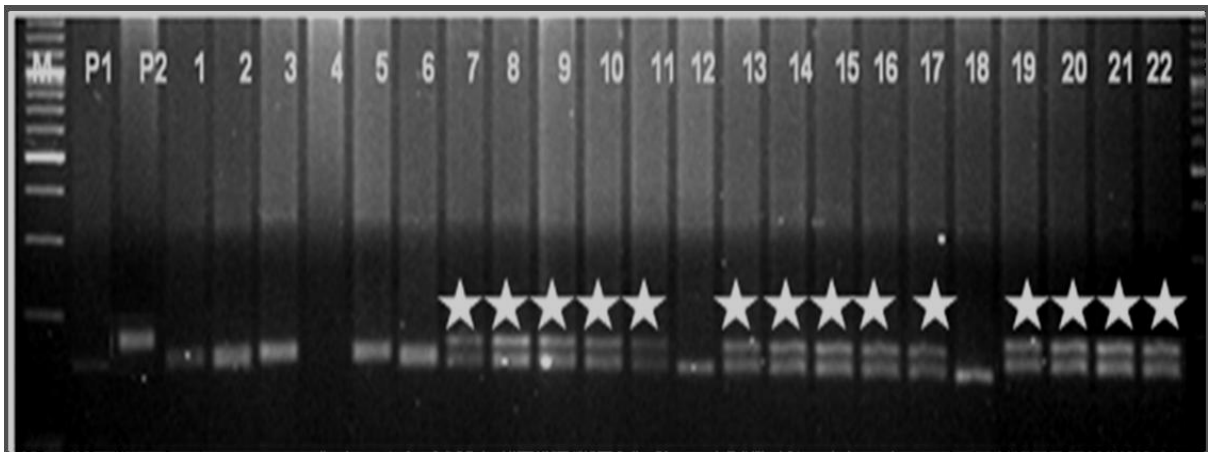

Fig. 2. Identification of opaque2 heterozygotes in the BC_1F_1 population using SSR marker phi057. Lane: 1=50bp ladder; P1=non QPM parent; P2=QPM donor parent; 1 to 22= BC_1F_1 individuals: * Heterozygotes

opaque2 introgressed maize lines follow the segregation pattern as per Mendelian inheritance (Gupta *et al*., 2013; Marija *et al*., 2016; Hossain *et al*., 2018; Adunola *et al*., 2019). Feng *et al*. (2015) and Liu *et al*. (2015) achieved high recovery of recurrent parent genome in marker assisted background selection using SSR markers in just two backcross generations. Similarly, high recovery of recurrent parent genome was reported in the studies conducted by Gupta *et al*. (2013) and Pukalenthy *et al*. (2019). They also reported that the *opaque2* introgressed maize lines had similarity for morphological traits like plant architecture and ear- and grain- characteristics with their corresponding recurrent parent.

Phenotypic selection on the basis of the ear phenotypes of each recurrent parent was carried out in order to further aid in the recovery of the recurrent parent phenotype. The kernels of BC_2F_2 plants with varied level of modification *viz*., 0, 25, 50, 75 and 100% opaqueness was observed through a light table box. Only those kernels that had 25% opaqueness were selected and forwarded to the next generation to fix the *o*2 in its homozygous recessive form. Similar phenotypic selection for kernel modification was carried out by Pukalenthy *et al*. (2019).

The effect of gene is studied through its expression by means of protein product that the gene encodes. In the present investigation, the effect of expression of *o2* gene on tryptophan concentration in maize kernel was quantified. Tryptophan content of BC_2F_4 generation was found to be ranged from 0.75 to 0.93% (**Table 2**). The minimum tryptophan concentration of 0.75 per cent

Table 2. Total protein content and per cent tryptophan in selected BC_2F_4 families of the cross BAJIM-08-26 x CML 169

Parent/Progeny	Total protein content (%)	Tryptophan in protein (%)
BAJIM-08-26 (Recurrent parent)	7.70	0.42
CML 169 (QPM donor)	8.75	0.99
B40-5-3	7.20	0.83
B42-3-1	7.80	0.87
B52-3-4	8.50	0.79
B76-22-5	8.75	0.75
B69-66-1	7.80	0.81
B69-66-2	7.80	0.85
B70-5-2	8.75	0.85
B70-5-5	8.75	0.75
B70-5-8	7.00	0.93
B75-4-2	7.00	0.72
B76-5-2	6.80	0.81
B76-22-2	7.80	0.92

Table 3. Performance of selected BC_2F_4 families of the cross BAJIM-08-26 X CML 169 for seed yield and other morphological trait

Parent/Progeny	Grain yield (q ha^{-1})	Days to 50% pollen shed	Days to 50% silking	Plant height (cm)	Cob placement height (cm)	Days to 75% maturity
BAJIM-08-26 (Recurrent parent)	103.47	57.50	60.00	199.38	105.03	103.28
CML 169 (QPM donor)	88.7	56.17	58.67	188.47	101.40	98.68
B40-5-3	92.6	56.83	59.33	212.57	114.40	104.15
B42-3-1	100.54	54.67	57.33	210.03	108.77	103.60
B52-3-4	85.58	57.50	60.17	191.43	92.23	102.30
B69-66-1	91.37	57.33	59.83	203.53	105.83	102.78
B69-66-2	89.06	57.50	60.00	206.33	109.03	104.38
B70-5-2	91.65	58.00	60.50	208.23	110.30	104.14
B70-5-5	77.96	50.00	52.50	192.45	96.73	92.93
B70-5-8	101.27	55.83	58.67	189.63	98.57	98.39
B75-4-2	80.43	58.83	61.17	198.87	108.03	101.47
B76-5-2	89.47	55.00	57.33	201.00	100.17	100.59
B76-22-2	102.75	57.33	60.50	207.07	107.77	102.68
B76-22-5	91.52	52.50	54.00	186.40	98.53	96.86
CD (5%)	13.41	2.84	2.88	22.23	13.46	3.61
CV (%)	7.22	2.50	2.42	5.48	6.39	1.78
Overall Mean	91.88	56.21	58.79	200.46	104.13	100.18

was reported in B70-5-5 and B76-22-5 lines while the maximum tryptophan concentration of 0.93 per cent was observed in B70-5-8 line of BAJIM-08-26 × CML169. The total protein in endosperm ranged from 7.0 to 9.6%.

The effect of gene is studied through its expression by means of protein product that the gene encodes. In the present investigation, the effect of expression of *o2* gene on tryptophan concentration in maize kernel was quantified and found to be ranged from 0.75 to 0.93% in progenies of BC_2F_4 generation. The total protein in endosperm ranged from 7.0 to 9.6%. These results are in line with the findings of Babu *et al*. (2005); Manna *et al*. (2005); and Tufchi *et al*. (2015).

Twelve selected BC_2F_4 progenies of the cross BAJIM-08-26 X CML 169 were evaluated for different agronomic traits. It was recorded that B76-22-2 (102.75 q ha^{-1}), B70-5-8 (101.27 q ha^{-1}) and B42-3-1(100.54 q ha^{-1}) were found to have similar grain yield with respect to recurrent parent (103.47 q ha^{-1}).

These lines were also evaluated for different agronomic traits and the data obtained for all the traits are represented in **Table 3**. Days to 50% silking in all the inbreds ranged from 57.33 to 61.17, days to pollen shed from 55.0 to 58.83, days to 75% maturity was ranged from 98.39 to 104.38. Similarly, the three lines homozygous for the target allele (*o2o2*) namely DBT 4-1-1/ 25-10/25-17/25-11, DBT 4-1-1/25-10/25-10/25-16 and DBT 4-1-1/25-10/25-17/25-13 developed by Pukalenthy *et al*. (2019) had similarity with the recurrent parent. Hossain *et al*. (2018) also reported that three MAS-derived inbreds and hybrids developed by them have been found to be identical to their non-QPM inbreds and hybrid.

Marker assisted selection in combination with phenotypic selection could greatly increase the conversion of normal maize into QPM. In the present study introgressed *opaque2* gene showed remarkable superiority in terms of various agronomic as well as biochemical traits over the normal maize. The promising QPM version developed in the present study can be used for the generation of a single cross hybrid of quality protein maize version.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the Department of Biotechnology, New Delhi (India) for financial aid to carry out the present investigation.

REFERENCES

Adunola, P.M., Akinyele, B.O., Odiyi, A.C., Fayeun, L.S. and Akinwale, M.G. 2019. Introgression of opaque-2 gene into the genetic background of popcorn using marker assisted selection. *Int. Res. J. Biotechnol.,* **5:** 10-18.

Babu, B.K., Agrawal, P.K., Saha, S. and Gupta, H.S. 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. [Cross Ref]

Babu, R., Nair, S.K., Kumar, A., Vekantesh, S. and Sekhar, J.C. 2005. Two generation marker aided backcrossing for rapid conversion of normal maize lines to quality protein maize (QPM). *Theor. Appl. Genet*., **111:** 888-897. [Cross Ref]

Babu, R., Nair, S.K., Prasanna, B.M. and Gupta, H. S. 2004. Integrating marker assisted selection in crop breeding: prospects and challenges. *Curr. Sci*., **87:** 607-619.

Danson, J., Mbogori, M., Kimani, M., Lagat, M., Kuria, A. and Diallo, A. 2006. Marker assisted introgression of *opaque*2 gene into herbicide resistant elite maize inbred lines. *Afr. J. Biotechnol.,* **5:** 2417-2422.

Feng, F., Wang, Q., Liang, C., Yang, R. and Li, X. 2015. Enhancement of tocopherols in sweet corn by marker-assisted backcrossing of ZmVTE4. *Euphytica,* **206:** 513–521. [Cross Ref]

Gupta, H.S., Agarwal, P.K., Mahajan, V., Bisht, G.S. and Kumar, A. 2009. Quality protein maize for nutritional security: rapid development of short duration hybrids through molecular marker assisted breeding. *Curr. Sci.,* **96**:230-237.

Gupta, H.S., Babu, R., Agarwal, P.K., Mahajan, V., Hossain, F. and Thirunavukkarasu, N. 2013. Accelerated development of quality protein maize hybrid through marker-assisted introgression of opaque-2 allele. *Plant Breed.,* **132** :77-82. [Cross Ref]

Hospital, F., Chevalet, C. and Mulsant, P. 1992. Using markers in gene introgression programs. *Genetics,* **132:** 1199-1210. [Cross Ref]

Hossain, F., Muthusamy, V., Pandey, N., Vishwakarma, A.K. and Baveja, A. 2018. Marker-assisted introgression of opaque2 allele for rapid conversion of elite hybrids into quality protein maize. *J. Genet.,* **97:** 287–298. [Cross Ref]

Jompuk, C., Cheuchart, P., Jompuk, P. and Apisitwanich, S. 2011. Improved tryptophan content in maize with Opaque-2 gene using Marker Assisted Selection (MAS) in backcross and selfing generations *Kasetsart J.,* **45:** 666-674.

Kjeldahl, J. 1883. New method for the determination of nitrogen in organic substances. *Fresen. J. Anal. Chem*., **22:** 366– 82.

Kostadinovic, M., Ignjatovic, M.D., Stankovic, G., Vancetovic, J. and Ristic, D. 2015. Marker Asissted Backcrossing for Incorporation of the Opaque2 gene into a Standard Maize Inbred Line. *J. Int. Sci. Publ. Agric. Food,* **2:** 199-205.

Krivanek, A., Groote, H., Gunaratna, N., Diallo, A. and Freisen, D. 2007. Breeding and disseminating quality protein maize for Africa. *Afr. J. Biotechnol.,* **6:** 312-324.

Larkins, B.A., Wu, Y., Song, R. and Messing, J. 2017. Maize seed storage proteins. In: Maize kernel development, B.A. Larkins (Ed.), CAB International, Wallingford, pp. 175–189. [Cross Ref]

Lekhi,Prince. Gill R.K., Kunal and Kaur S. 2018. Identification of molecular marker linked to mungbean yellow mosaic virus (MYMV) resistance in *Vigna radiata* (L.) Wilczek. *Electronic Journal of Plant Breed*ing, **9**(3):839-845. [Cross Ref]

Liu, L., Jeffers, D., Zhang, Y., Ding, M., Chen, W., Kang, M.S. and Fan, S. 2015. Introgression of the crtRB1 gene into quality protein maize inbred lines using molecular markers. *Mol. Breed.,* **35:** 154-166. [Cross Ref]

Magulama, E.E. and Sales, E.K. 2009. Marker-assisted introgression of opaque 2 gene into elite maize inbred lines. *USM R & D.,* **1**7: 131-135.

Manna, R., Okello, D.K., Imanyhowa, J., Pixley, K. and Edema, R. 2005. Enhancing introgression of opaque- 2 trait into elite maize lines using simple sequence repeats. *Afr. J. Crop Sci.,* **13:** 215-226.

Marija, K. Dragana, I.M. Jelena, V. Danijela, R. Sofija, B. Goran, S. and Snezana, M.D. 2016. Development of high tryptophan maize near isogenic lines adapted to temperate regions through marker assisted selection - impediments and benefits. *PLoS one,* **11:** 1-17. [Cross Ref]

Mertz, E.T., Jambunathan, R. and Misra, P.S. 1975. Protein quality. Agricultural Research Station Bullutein, Purdue University, USA.

Misra, P.S., Mertz, E.T., Glover, D.V. and Barbosa, H.M. 1972. Endosperm protein synthesis in maize mutants with increased lysine content. *Science*, **176**: 1425–1427. [Cross Ref]

Murray, M.G. and Thompson, W.F. 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.,* **8:** 4321-4325. [Cross Ref]

Nelson, O.E., Mertz E.T. and Bates, L.S. 1965. Second mutant gene affecting the amino acid pattern of maize endosperm proteins. *Science*, **150**: 1469–1470. [Cross Ref]

Nyakurwa, C.S., Gasura, E. and Mabasa, S. 2017. Potential

for quality protein maize for reducing protein energy undernutrition in maize dependent sub-saharan African countries: A Review. *Afr. J. Crop Sci.,* **25:** 521–537. [Cross Ref]

Pomeranz, Y., Martin, C.R., Traylor, D.D. and Lai, F.S. 1984. Corn hardness determination. *Cereal Chem.,* **61:** 147-156.

Pukalenthy, B., Manickam, D., Chandran, S., Adhimoolam, K., Sampathrajan, V., Rajasekaran, R., Arunachalam, K., Ganapathyswamy, H., Chocklingam, V., Muthusamy, V., Hossain, F. and Natesan, S. 2019. Incorporation of opaque-2 into 'UMI 1200', an elite maize inbred line, through marker-assisted backcross breeding. *Biotechnol. Biotech. Eq.,* **33:** 144-153. [Cross Ref]

Ren,Y., Yobi, A., Marshall, L., Angelovici, R., Rodriguez, O. and Holding, D.R. 2018. Generation and evaluation of modified Opaque-2 popcorn suggests a route to quality protein popcorn. *Front Plant Sci.,* **9:** 1803-1814. [Cross Ref]

Surender, M., Shetti, P., Sagare, D.B., Durga, R.C.V., Jabeen, F., Sudarshan, M.R. and Sokka, R.S. 2017. Development of QPM version of DHM117 maize hybrid using marker assisted selection. *Inter. J. Curr. Microbiol. Appl. Sci.,* **6**:3275–3289. [Cross Ref]

Tripathy, S.K., Ithape, D.M., Maharana, M. and Prusty, A.M. 2017. Quality protein maize (QPM): genetic basis and breeding perspective. *Tropical Plant res.,* **4**:147-52. [Cross Ref]

Tsai, C.Y. and Dalby, A.1974. Comparison of the effect of shrunken-4, opaque-2, opaque-7, and floury-2 genes on the zein content of maize during endosperm development. *Cereal Chem*., **51**: 825–829.

Thakur, N., Kumari, R., Prakash, Jay., Sharma, J.K., Singh, N. and Lata, S. 2015. Evaluation of elite maize genotypes (*Zea mays* L.) for nutritional traits. *Electronic Journal of Plant Breeding*, **6 (1)**: 350-354.

Tufchi, M., Rashmi, I., Jha, S.K. and Singh, N.K. 2015. Effect of opaque-2 allele on accumulation of tryptophan among backcross-derived introgressed progenies of maize. *Indian J. Genet.,* **75:** 453-458. [Cross Ref] .

Vivek, B.S., Krivanek, A.F., Palacios, R.N., Twumasi, S. and Diallo, A. 2008. Breeding quality protein maize: protocols for developing QPM cultivars. International maize and wheat improvement centre, Mexico .

Yadav, O.P., Hossain, F., Karjagi, C.G., Kumar, B., Zaidi, P.H. and Jat, S.L. 2015. Genetic improvement of maize in india: retrospect and prospects. *Agric. Res.,* **4:**325–338. [Cross Ref]

Zunjare, R.U., Hossain, F., Muthusamy, V., Baveja, A., Chauhan, H.S., Bhat, J.S., Thirunavukkarasu, N., Saha, S. and Gupta, H.S. 2018. Development of Biofortified Maize Hybrids through Marker-Assisted Stacking of β-Carotene Hydroxylase, Lycopene-ε-Cyclase and Opaque2 Genes. *Front Plant Sci.,* **9:** 1-12. [Cross Ref]