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Molecular analysis of the F_4 progenies obtained through pollen selection for heat tolerance in maize (*Zea mays*)

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Maize (*Zea mays* L.) is grown in diverse environmental conditions where various kinds of environmental stresses impair growth and development at different stages (Payero *et al.* 2006, Cattivelli *et al.* 2008) which directly decreases crop productivity. It is reported that, 1ºC increase in global mean temperature could reduce global yields of maize by 7.4% which is highest among the cereals (Zhao *et al.* 2017). High temperature over 35°C at the flowering stage reduces pollen germination, pollen tube elongation, fertilization abilities and kernel development, resulting in longer anthesis-silk interval, lower yield and harvest index (Fahad *et al.* 2017). Maize is highly susceptible to heat stress particularly at reproductive stage, specifically the male reproductive system (Thakur *et al.* 2010, Prasad *et al.* 2017, Lizaso *et al.* 2018, Lohani *et al.* 2020). It has been shown that, the prolonged exposure to high temperature reduced the amount of pollen shed and its viability (Pacini and Dolferus 2019). The life cycle of angiosperms mainly consists of two parts, viz. gametophytic and sporophytic. The gametophytic selection is found to be very effective in the selection of biotic and abiotic stress tolerance and can be easily combined with conventional method to obtain higher efficiency (Shobha Rani and Ravikumar 2006). The populations improved through gametophytic selection appear to be more promising particularly for complex trait like heat tolerance as the pollen selection allowed the selection of favorable allelic combinations that would hardly be detected at the sporophytic level (Singh *et al.* 2020). A few investigations have demonstrated the persistence of positive response to gamatophytic selection in the succeeding generations (Ravikumar *et al.* 2007, Mohapatra *et al.* 2020). We have demonstrated that both combination of gametophytic and sporophytic selection has resulted in developing the lines with higher level of heat tolerance in maize (Singh *et al.* 2020). In the present study, evidence is provided using molecular markers that the inbred lines developed through different cycles of pollen selection are genetically different from the lines developed without pollen selection.

The present study was carried out at Department of Plant Biotechnology, University of Agricultural Sciences, Gandhi Krishi Vignana Kendra, Bengaluru, Bengaluru, Karnataka during 2017–20. Two parental lines contrasting for heat stress tolerance, viz. BTM4 (susceptible) and BTM6 (tolerant) were crossed to produce F_1 hybrid seeds (Singh *et al.* 2017). Further the F_1 plants were selfed and advanced to F_4 generation through different cycles of pollen selection for heat tolerance as described further. One set F_1 plants were selfed using heat stressed pollen grains (36˚C for 3) to produce pollen selected F2 (G) population. The F_2 (G) plants were advanced to F_3 generation by selfing using heat stressed pollen grains producing pollen selected F_3 (GG) progenies. The F_3 (GG) progenies were advanced to F_4 generation (GGG) by selfing using heat stressed pollen grains. Another set of F_2 (G) progenies were advanced to F_3 (GC) by selfing without heat stressed pollen grains. The F_3 (GC) progenies were advanced to F_4 generation (GCG) by selfing using heat stressed pollen grains. Alternatively, another set of F_1 plants were selfed using normal pollen grains (no heat stress treatment) to produce F_2 (C) plants. Subsequently, F_2 plants were selfed using normal pollen grains to produce F_3 (CC) progenies followed by F_4 (CCC) progenies. The details of the methodology followed for heat stress treatment to pollen grains is detailed in Singh *et al.* (2020). The three groups of F_4 progenies used for the present study are GGG (pollen selection for heat tolerance in F_1, F_2 and F_3 generation); GCG (pollen selection for heat tolerance only in F_1 and F_3 generation) and; CCC (no pollen selection for heat tolerance in F_1, F_2 and F_3 generation).

Five F_4 progenies from each group, totally 15 progenies were randomly selected for the present study. The selected progenies along with parents (BTM4 and BTM6) were grown in small cups and the DNA was isolated from the

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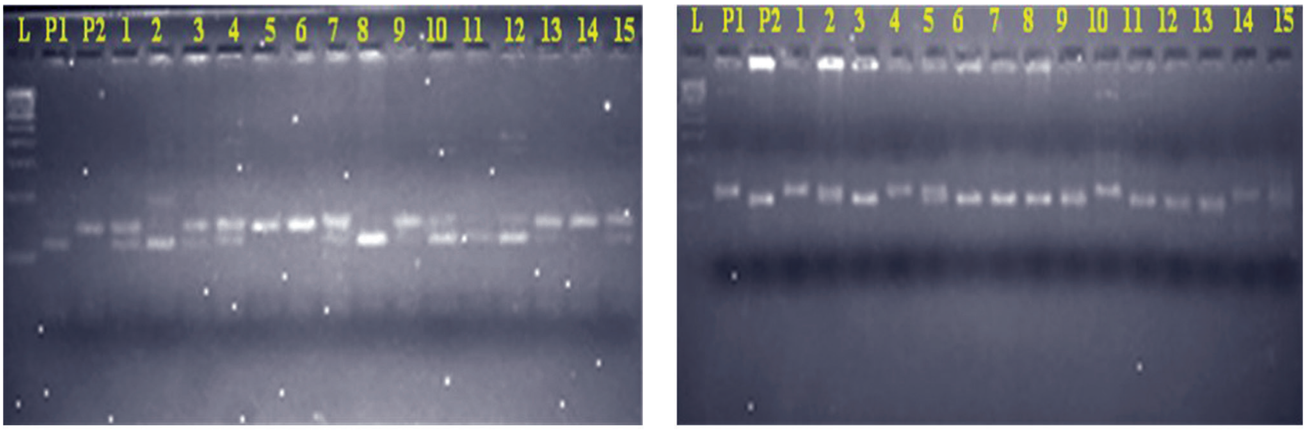
Table 1 List of polymorphic SSR primers used to study genetic differences in three groups of F_4 progenies in maize

Primer	Forward (5’-3’)	Reverse (5’-3’)
bnlg1526	ACGAGCGAGTGGAGAATAGG	AGCCCAGTACGTGGGGTC
phi 96342	GTAATCCCACGTCCTATCAGCC	TCCAACTTGAACGAACTCCTC
bnlg1047	ATGGAGATGGAGGAGAGAGAGA	GATGCGGCGATGGCTAA
bnlg 1792	CGGGAATGAATAAGCCAAGA	GCGCTCCTTCACCTTCTTTA
bnlg 1662	GCACCCACATGAAGTATCCC	TTGTTTTTGCAGTGCCTCAG
umc1894	TTTCTCATGACATTGCAAGCATCT	TATGCAAAAGGTGAGCCTGCTTAT
bnlg 1909	CCTGACCCTGTTCCTGAAAA	GTGTGTCTGGAGCTGTTCGA
umc1082	CCGACCATGCATAAGGTCTAGG	GCCTGCATAGAGAGGTGGTATGAT
umc 2196	ATCCTCTCCCATCTCTCGGTCT	CCTAGACCAGCTTGGTGACTCAG
bnlg 1927	TTTTTTTGTAAGCGATCCGG	GATGAATCTGCGTCCGTCTT
umc 1781	CAAAAGGTGCAACTGTAATTGCCT	ATGAGGCGAAGCCAAGAAAAAGT
umc 1329	CCTCTCACATCTCCTCTCCCCT	GTGTCGGTGTAGGTCTCCGTCTT
umc 1766	ACAAGAAGGAATCGAGAGCAAATG	CTTCGGGATGGAGTCGTAGTTC
bnlg 1144	TACTCGTCGTGTGGCGTTAG	AGCCGAGGCTATCTAACGGT
umc 1381	CTCTAGCTACGAGCCTACGAGCA	CCGTCGAGTCAACTAGAGAAAGGA
umc 1080	GAGGAGAAAAGGAGATGGAAAAGC	AGATGCCGCAGAAGATTCTAAACA

leaves of the young seedlings by following C-TAB method (Doyle and Doyle 1987). Totally 180 simple sequence repeat (SSR) primers were used to screen for parental polymorphism, out of which 16 primers were found to be consistently polymorphic on agarose gel (Table 1). The 16 polymorphic SSR primers were used to examine the allelic pattern of parents in F_4 progenies. The PCR amplification was carried out in a Thermal Cycler (Master cycler gradient, Eppendorf, Hamburg, Germany) in a PCR reaction mixture containing 6.3 µl of DNase free water, 1 µl of 1X Taq DNA buffer (with $MgCl_2$), 0.4 µl of dNTP, 1.0 µl of primer (forward and reverse), 0.3 µl of Taq polymerase (1U/ µl) and 1.0 µl sample DNA. The PCR amplification for each primer was performed at 95°C for 5 min, 95°C for 1 min, 45–55°C* for 1.5 min (*based on annealing temperature), 72°C for 2 min and 72°C for 8 min with 38 cycles. The PCR product was separated using 3.5% agarose gel containing ethidium bromide (10 mg/ml). Gel scoring was done by considering the banding pattern of each individual F_4 progenies (Fig 1). Three types of banding pattern for each primer were observed as homozygous male, homozygous female and heterozygote in the progenies.

One hundred eighty SSR primers were used to screen two parental lines BTM4 and BTM6 out of which 16 primers (8.89%) were found to be polymorphic among parents. The extent of polymorphism observed in the present study was lower and found similar to earlier studies in maize (Yumurtaci *et al.* 2017). Together for all the 16 primers, the homozygous male, homozygous female and heterozygotes were determined for GGG, GCG and CCC groups of inbred



a) bnlg 1526

b) bnlg 1792

Fig 1 The agarorse gel depicting parental allelle distribuion in 15 F_4 progenies of maize.
(a) SSR primer, bnlg 1526; (b) SSR primer, bnlg 1792
L, 100 bp ladder; P1, BTM4; P2, BTM6; Lane 1–5, GGG; Lane 6–10, GCG; Lane 11–15, CCC F_4 lines.

Table 2 Parental type alleles recorded among three different groups of F_4 progenies of maize as revealed by SSR markers

Allele type	Number of parental alleles in F_4 progenies		
	GGG	GGC	CCC
Homozygous male (Heat tolerant parent-BTM6)	46 (57.5)	38 (47.5)	33 (41.25)
Homozygous female (Heat susceptible parent-BTM4)	21 (26.25)	26 (32.5)	27 (33.75)
Heterozygote	13 (16.25)	16 (20)	20 (25)

Values in parenthesis are percentage of parental lines in F_4 progenies.

separately. The number of homozygous male type of allele was highest, i.e. 46 (57.5%) in GGG progenies followed by 38 (47.5%) in GCG and the least, i.e. 33 (41.25%) in CCC F_4 progenies (Table 2). GGG progenies developed through three cycles of pollen selection for heat tolerance carried more number of homozygous male parental alleles indicating selection pressure applied for heat tolerance at gametophytic stage (pollen stage) resulted in increased number of male parent alleles in progeny. The number of homozygous female parental banding types observed was highest, 27 (33.75%) in CCC and lowest, 21 (26.25%) in GGG progenies. The results indicated that, the cyclic pollen selection pressure for heat tolerance in three generations (GGG progenies) lead to the reduction of female alleles.

The effect of pollen selection for heat tolerance in F_1 generation on the segregation of SSR markers was studied in the F_2 population of maize (Mohapatra *et al.* 2020, Singh *et al.* 2020). They found that, where there is no pollen selection, the expected monogenic segregation was observed in the control population (no pollen selection). On the contrary, significant deviation from the expected monogenic ratio was observed in the pollen selected F_2 population. In the present study, GGG F_4 progenies developed through three cycles of pollen selection for heat tolerance carried more frequency of male parental alleles compared to CCC and GCG populations. The results indicate that pollen selection has shifted the allelic frequencies in the desirable directions (Ottaviano *et al.* 1988). The effectiveness of gamete selection for increasing the frequency of resistant alleles in the resultant population was reviewed in tobacco (Touraev *et al.* 1995), maize (Frascaroli and Songstad 2001) and chickpea (Ravikumar *et al.* 2007; Babu and Ravikumar 2009). The F_3 and F_4 progenies were also tested for heat tolerance observed under field and laboratory condition and the GGG F_4 progenies were found to be more tolerant to heat stress demonstrating the practical benefits of pollen selection for a complex trait such as heat tolerance in maize (Singh *et al.* 2020, Singh *et al.* 2022). The cyclic pollen selection was found useful particularly for quantitative traits like osmotic stress tolerance in sorghum (Ravikumar *et al.* 2003) and demonstrated the transmission of the selected trait from pollen generation to progeny (Patil *et al.* 2006). Hence, the combination of gametophytic selection and conventional sporophytic selection should be considered as an efficient tool in population improvement programs to achieve higher levels of tolerance to biotic and abiotic stresses relatively in short time.

SUMMARY

In the present study, three sets of F_4 progeny lines developed through different cycles of pollen selection for heat tolerance were studied for the genetic differences using 16 SSR markers during 2017–20 at Department of Plant Biotechnology, University of Agricultural Sciences, Gandhi Krishi Vignana Kendra, Bengaluru, Karnataka. Three groups of F_4 progenies used for the study are GGG (pollen selection for heat tolerance in F_1, F_2 and F_3 generation); GCG (pollen selection for heat tolerance only in F_1 and F_3 generation); CCC (no pollen selection for heat tolerance in F_1, F_2 and F_3 generation). Five randomly selected F_4 lines of the cross of heat stress susceptible BTM4 and heat tolerant BTM6 represented each group. The three groups differed significantly for the number of male parent alleles as evidenced by SSR markers. The F_4 (GGG) progenies had significantly more number of male parent type alleles compared to F_4 (GCG) and F_4 (CCC) lines. The F_4 (CCC) lines recorded more number of female alleles compared to other F_4 (GGG and GCG) lines. The effectiveness of pollen selection for heat tolerance towards increasing the frequency of male parent alleles and their transmission to the succeeding progenies has been demonstrated in the present study.

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