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Original Research Paper

Genetic inheritance and identification of molecular markers linked to male sterility in African marigold (*Tagetes erecta* L.)

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ABSTRACT

The demand for hybrid varieties of African marigold (*Tagetes erecta* L.) is on the rise due to the increased productivity and uniformity exhibited by F_1 hybrids. To develop hybrids in marigold, male sterile line is essential, as emasculation is complex due to the unique flower structure of marigold. Inheritance of petaloid male sterility was investigated in a seed propagated line across six generations (P_1, P_2, F_1, F_2, BC_1 and BC_2), that confirmed a single dominant gene governing petaloid male sterility over multiple seasons. The marker CPSSR-39 exhibited clear and consistent segregation, following a Mendelian ratio 1:1 in F_1 which is in accordance with the genes governing petaloid male sterility. This is the first report of genic petaloid male sterility with a linked marker marking a significant advancement in heterosis breeding of marigold.

Keywords: African marigold, Mendelian genetics, male sterility, petaloid flower, SSR markers

INTRODUCTION

Among flower crops, marigold (*Tagetes erecta* L.), family Asteraceae, is gaining popularity due to the rising demand for loose flowers in the market as well as an industrial input for the extraction of carotenoids. There is a global market for carotenoids as food colourants, animal feed and nutraceuticals (Berman et al., 2015). Some carotenoids are chemically synthesized, while, marigold is the major natural source of carotene (Rodrigues et al., 2019).

Marigold flower has a capitulum inflorescence consisting of ray and disc florets. Functional anthers in marigold are hidden within disc florets in the centre of the flower, making emasculation a difficult process (Fig. 1). Male sterility is the major approach to avoid emasculation to cut down the labour, cost of hybrid seed production and to ensures varietal purity (Du et al., 2020). In marigold, two types of male sterility were reported. The absence of ray florets is associated with apetaloid male sterility (Gupta et al., 1999; Tejaswini et al., 2016a), while, flowers with only ray florets are associated with petaloid male sterility (Tejaswini et al., 2016a) (Fig. 1).

In marigold, apetaloid male sterility is reported to be controlled by a single recessive gene (Gupta et al.,

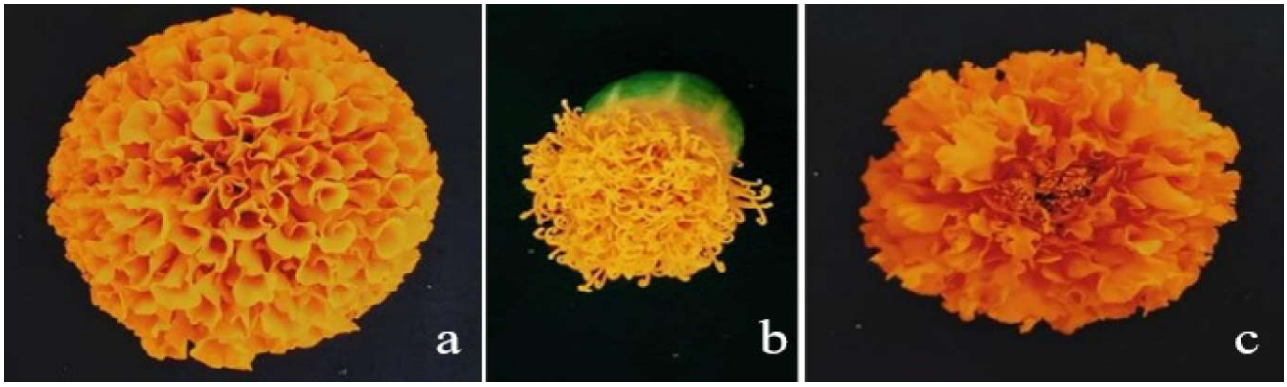


Fig. 1: Flower forms in african marigold (a) petaloid sterile flower with only ray florets, (b) apetaloid sterile flower with only gynoecium, (c) hermaphrodite flower with both ray and disc florets

1999; Tejaswini et al., 2016a), while, petaloid are reported to be cytoplasmic inheritance and maintained by vegetative propagation (Kumar et al., 2017; Tejaswini et al., 2016b). In the genic male sterility (GMS), the segregation occurs at 1:1 ratio, necessitating the removal of 50% fertile plants, which is time and labour-consuming and also difficult to identify prior to flowering. Identifying molecular markers tightly linked to the male sterility locus helps in early and accurate identification of male sterile genotypes at the seedling stage itself and has been identified in apetaloid male sterility system in marigold (He et al., 2010; Asha et al., 2019a).

Though sufficient work has been reported with apetaloid sterility in marigold, there is not much information available on seed-propagated petaloid male sterility either in terms of gene action or in terms of a molecular marker association. Hybrids produced from petaloid male sterile lines exhibit good combining ability for increased flower weight and biochemical components compared to apetaloid sterile lines (Santosh et al., 2018). The present study tried to unravel the gene action associated with seed-propagated petaloid male sterility and the marker associated with that, in an attempt to widen the genetic base of male sterility in marigold.

MATERIALS AND METHODS

Plant material

Plant materials for the present study were selected from the progeny population of an ongoing marigold breeding program at ICAR-Indian Institute of Horticultural Research, Bengaluru, India. Three petaloid male sterile lines *viz*., IIHRMOP 1111, IIHRMOP 22 and IIHRMOP 228 were evaluated. All these are of two type lines with sterile and fertile plants maintained by intercrossing. Observations on the number of fertile and sterile plants resulting from intercrossing as well as selfing were recorded. For confirmation studies line IIHRMOP 1111 was selected and to ensure the stability of the segregation, repeated intercrossing between sterile and fertile plants along with the selfing of fertile plants for three consecutive seasons, was performed.

The F_1, F_2, BC_1 and BC_2 were developed in combination with stable fertile variety Pusa Narangi Gainda. In all the generations, plants were phenotyped for male sterility based on floral characteristics and number of fertile and sterile plants were recorded.

DNA isolation

The DNA was isolated from the young leaves of both the parents, and from the F_1 population (petaloid sterile × PNG; N=96) by using the CTAB method as described by Doyle & Doyle (1990) with some minor modifications, including RNAase treatment. The quality was analyzed by using 0.8% agarose gel and quantity by using a UV-Vis spectrophotometer. The final DNA concentration was adjusted to 60 ηg/ul.

Genotyping

The eleven SSR microsatellite markers linked to apetaloid male sterility *viz*., CPSSR4, CPSSR7,

Table 1 : The sequences of markers used in the present study

Markers	Primer sequence (5' – 3')	
	Forward primer	Reverse primer
CPSSR 4	TCCACATCAAATTCTTGGTCCCT	GGGGAGGGTCGTTGCATATT
CPSSR 7	CGTGATGTCGAAACGTTGTGG	TGAAGGTGGTGGTGCCTTTT
CPSSR 11	AGAGAGAGAGACCACTGTTGT	TCACAACATCACAGCTCAACAC
CPSSR 16	CCATTAAAGGGCTCGACGGA	GGACTTGCTCCGCTACCTAC
CPSSR 26	GCTGTTGGAGCCACTGATCT	ACATCAATCCCTACAAAACCCT
CPSSR 33	CAATTTTCGTTCCGGCTGCA	GAGCATGTTGCCTCAGAGGT
CPSSR 37	ACCCGTACCCAATCCCAATT	GCAGCACTACTACAACCACCA
CPSSR 39	ACTCACGGGAGGAGAAATGC	CAGAAGCAGAGACCGGTCTG
CPSSR 47	TCGGGGAGATGTCTGAATTTGG	CGTCACGCATAAACGAATGT
CPSSR 53	TGGGATGATCTGGGAGCTGA	AGTGTCCAACCAAAAGCCCTA
CPSSR 66	CGATGACGTTGACGGACTTTG	AGGCCGAATTGAAGGTGATGT

CPSSR11, CPSSR16, CPSSR26, CPSSR33, CPSSR37, CPSSR39, CPSSR47, CPSSR53 and CPSSR66 shortlisted in the previous report (Asha et al., 2019a) were used in the present study (Table 1). The parental polymorphic SSR markers were utilized for screening the F_1 population to evaluate the co-segregating nature and confirm the linkage with sterile loci.

PCR analysis

Genotyping was carried out in an Eppendorf Thermocycler (Eppendorf master cycler, Germany)

Table 2 : Segregation pattern observed in intercross and selfed progeny of identification of stable segregating petaloid male sterile line

Sl. No.	Intercross and selfing of petaloid sterile lines	No. of sterile plants	No. of fertile plants observed	Expected chi-square ratio observed	Chi-square value (sterile:fertile)	Probability
1	**IIHRMOP 1111**					
a	IIHRMOP 1111-s X IIHRMOP 1111-f	22	21	1:1	0.02	0.87
b	Self of IIHRMOP 1111-f	-	25	0:1	-	-
2	**IIHRMOP 22**					
a	IIHRMOP 22-s X IIHRMOP22-f	19 15	1:1	0.47	0.49	-
b	Self of IIHRMOP 22-f	-	8	0:1	-	-
3	**IIHRMOP 228**					
a	IIHRMP 228-s XIIHRMOP 228-f	15	23	1:1	1.68	0.19
b	Self of IIHRMOP 228-f	-	5	0:1	-	-

* s-sterile; f-fertile

Table 3 : Segregation pattern observed in intercross and selfed population of petaloid male sterile line IIHRMOP 1111 (IIHRMOP 1111-s + IIHRMOP 1111-f) in different seasons

Sl. No.	Intercross and selfing of petaloid sterile lines	No. of sterile plants	No. of fertile plants observed	Expected chi-square ratio observed	Chi-square value (sterile:fertile)	Probability
Season-1 (summer)						
a	IIHRMOP1111-s XIIHRMOP 1111-f	27	23	1:1	0.32	0.57
b	Self of IIHRMOP 1111-f	-	35	0:1	-	
Season-2 (rainy)						
a	IIHRMOP1111-s XIIHRMOP 1111-f	63	55	1:1	0.54	0.46
b	Self of IIHRMOP 1111-f	-	125	0:1	-	
Season-3 (winter)						
a	IIHRMOP 1111-s XIIHRMOP 1111-f	25	28	1:1	0.16	0.68
b	Self of IIHRMOP 1111-f	-	45	0:1	-	

* s-sterile; f-fertile

with an initial denaturation at 94ºC for 2 minutes, followed by 35 cycles; each cycle consisting of denaturation at 94ºC for 45 seconds, primer annealing at 60ºC for 45 seconds and primer extension at 72ºC for 45 seconds and a final extension for 10 minutes at 72º C and hold at 4ºC.

Statistical analysis

Phenotypic and genotypic data were analyzed by the Chi-square test to check the co-segregation and to determine the goodness–of–fit (Quinn & Keough 2002). Genotypic data were subjected to single marker–ANOVA to determine an independent assortment of markers with that of phenotype. The statistical analysis was performed using Statistical Analysis System Version 9.3 software (SAS, 2012).

RESULTS AND DISCUSSION

Identification of the stable genic male sterile lines

The intercrossing between fertile and sterile plants in all three petaloid lines (IIHRMOP 1111, IIHRMOP 22 and IIHRMOP 228), resulted in progenies with fertile and sterile plants with the segregation ratio of 1:1 while selfing of fertile plants resulted in progenies consisting of all fertile plants (Table 2). Inter-cross and selfing performed over three different seasons in the selected petaloid male sterile line IIHRMOP 1111 confirmed the stability (Table 3).

Genetics of male sterility in petaloid male sterile system

The F_1 resulting from IIHRMOP 1111(P_1) crossed with Pusa Narangi Gainda (P_2), segregated in the ratio of 1:1. All 210 plants of F_2 were observed to be fertile, while BC_1, and BC_2 plants segregated into a ratio of 1:1 (Table 4).

Molecular markers for petaloid sterility

The parent IIHRMOP 1111 and Pusa Narangi Gainda were assessed for polymorphism with eleven molecular markers and CPSSR39 was found to be polymorphic which could differentiate sterile and fertile genotypes.

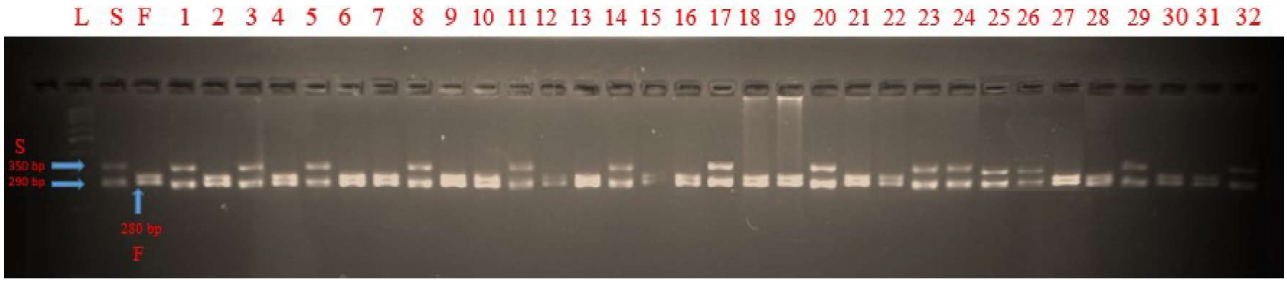
Validation of CPSSR-39 association with sterility trait

The PCR amplification using CPSSR 39 marker in petaloid sterile line resulted in two amplicons with size 350 bp and 290 bp and a single amplicon with size 280 bp in fertile pollen parent Pusa Narangi Gainda. This showed a clear polymorphism between the two parents. Genotyping of 96 individual population of the F_1 population using CPSSR 39 resulted in a heterozygous banding pattern in 40 sterile individuals and a homozygous banding pattern in 56 fertile individuals with the same amplification PCR ampliconsizes as of parents, co-segregating in accordance with the phenotype of each individual as

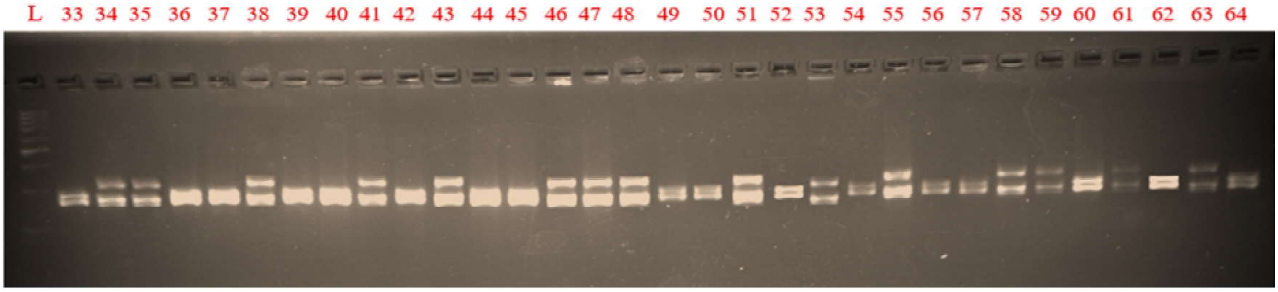
Table 4 : Segregation pattern observed in all the generations developed in crossing program between IIHRMOP 1111 and Pusa Narangi Gainda for confirmation of genes involved in petaloid male sterility

Initial material maintained/crossed			Resulting progeny				Chi-square value	Probability
Lines/varieties used in crossing	Genotype (Sterile: Fertile)	Gene ration	Expected chi-square ratio (sterile: fertile)	Expected genotypic ratio (sterile: fertile)	Number of sterile plants-observed	Number of fertile plants-observed		
IIHRMOP 1111 (IIHRMOP 1111-s + IIHRMOP 1111-f)	1Pp:1pp	P_1	1:1	1Pp:1pp	25	28	0.16	0.68
Pusa Narangi Gainda	0:pp	P_2	0:1	0:pp	0	45	-	-
IIHRMOP 1111-s X PNG	Pp x pp	F_1	1:1	1Pp:1pp	40	56	2.66	0.10
Self of fertile F_1	pp x pp	F_2	1:0	pp:0	0	210	-	-
IIHRMOP 1111-s X Fertile F_1	Pp x pp	BC_1	1:1	1Pp:1pp	34	24	1.72	0.18
Sterile F_1 X PNG	Pp x pp	BC_2	1:1	1Pp:1pp	21	24	0.2	0.65

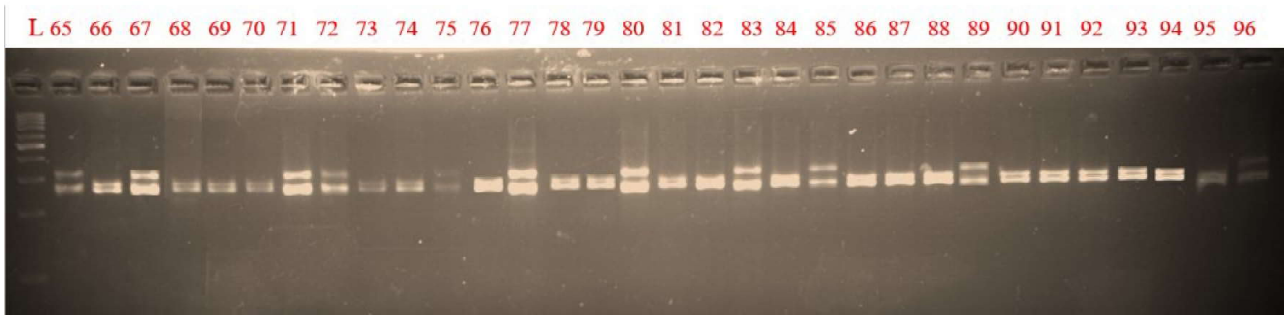
*s: sterile; f: fertile



(i) The PCR amplification of CPSSR 39 for petaloid F_1 population with sterile, fertile parent and 1-32 individuals



(ii) The PCR amplification of CPSSR 39 for petaloid F_1 population 33-64 individuals



(iii) The PCR amplification of CPSSR 39 for petaloid F_1 population 65-96 individuals

Fig. 2 : PCR amplification of CPSSR 39 with sterile, fertile parents and F_1 population of 1-96 individuals

per expectation; differentiating sterile and fertile individuals in 1:1 ratio (X^2 $p<0.05$, 2.66) (Fig. 2: i, ii, iii). The segregation of the polymorphism fitted into the expected 1:1 ratio with a probability of 0.10% confirms the linkage of this markers to petaloid male sterility. The single marker–ANOVA revealed the association between SSR marker with sterility in a segregated F_1 population of marigold at a 1% level of significance with an F value of 133. Since CPSSR 39 followed the mendelian segregation ratio, we presume that CPSSR39 can be efficiently used for the selection of parents in marker-assisted breeding programs.

The observed segregation pattern (Table 3 & 4), including the 1:1 ratio under intercross and the presence of 100% fertile plants in the selfed progeny of fertile plants, suggested that petaloid sterile plants are heterozygous (*Pp*), while fertile plants are homozygous recessive (*pp*). Further, the segregation pattern observed from F_1, F_2, BC_1 and BC_2 confirms the single dominant gene governing petaloid male sterility in marigold. Since banding pattern by CPSSR 39 confirmed the expected genotypic segregation and 100% matching with the observed phenotype, it was considered as a linked marker for petaloid male sterility. This observation is consistent with the segregation of a single dominant gene (*Pp*) governing petaloid male sterility. Consequently, CPSSR 39 can be efficiently utilized for selecting sterile plants in hybrid seed production and in marker-assisted breeding programs. Irrespective of crops, most of the reported male sterility were either monogenic recessive or

dominant (Joshi & Nabi, 2018). More than one gene was reported to exist governing male sterility in different crops (Saxena et al., 2010).

Homeotic conversion of ray and disc florets into sepal and style-like structures corresponding to abnormality of androecium and differential expression of B-class genes in floral development have been reported in apetaloid male sterile flowers of *T. erecta* (Ai et al., 2016; He et al., 2010). Petaloid sterile flowers studied in the present work indicate the possible conversion of stamens into petals projecting *T. erecta* is an ideal material for the study of homeotic genes as well as serving as an important breeding material for hybrid seed production.

CONCLUSION

The present study confirmed petaloid male sterility in marigold being governed by a single dominant gene based on its inheritance pattern. Apetaloid and petaloid male sterility are the two distinct types of male sterility based on flower structure and the present study suggests the existence of two different genes responsible for the expression of structurally variable male sterility in marigold. Male sterility linked markers in the present study indicated the possibility of MAS and the potential use of CPSSR 39 in the hybrid breeding programme of marigold. In conclusion, this molecular marker linked to petaloid male sterility can be used to select desired sterile plants which saves both costs and time in hybrid seed production.

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