

Original Research Article

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## Validation of SCAR Marker Linked to Genic Male Sterility in Marigold: As a Forward Step towards Marker Assisted Breeding Programme

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### ABSTRACT

#### Keywords

Marigold, Genic Male sterility, SCAR marker, Linked marker, MAS, Validation, Apetalloid sterile lines

#### Article Info

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The identification of marker tightly linked to male sterility will greatly facilitate for marker assisted selection (MAS) breeding through accurate selection of parental lines in hybrid production. In the present study, to assess the efficiency of previously reported SCAR 4 marker for marker assisted selection was validated by screening the marker in a total of 226 F<sub>2</sub> mapping population derived from a cross between male sterile (IIHR10521AB) and a male fertile pure line (IIHRMY7) maintained at IIHR along with bulk segregant analysis. The results showed that the marker segregated in the F<sub>2</sub> population showing that it is linked to sterility locus. The marker was also validated by screening 12 different apetalloid male sterile lines maintained at IIHR, the results of amplification gave clear and similar band size amplicons present in parents in all the apetalloid sterile lines confirming that it is linked to male sterility and hence this study is significantly useful and can offer a powerful tool for the efficient selection in MAS breeding programmes in marigold.

### Introduction

Marigold (*Tagetes erecta* L.) is an ornamental plant belonging to the Compositae family. It is grown in many parts of India as well as throughout the World. Marigold is widely grown as a border plant in gardens. Different varieties and flowers are available in various shades of yellow, red, orange, dark orange, and orange brown. The two common species

of marigold, both annuals, are distinguished as African marigold (*T. erecta*) and French marigold (*T. patula*) and are native to Mexico and Gautemala and were popularly grown as a cut flower, loose flower, pot plant and as a bedding plant in garden and for its medicinal values (Sowbhagya *et al.*, 2004). Prospects of commercialization of marigold are increasing because of its hardy nature with ease to grow, low nutrient requirement and easy availability

of planting material. Many *Tagetes* species are known to yield aromatic essential oils which are known as Tagetes oils potential of being used as antimicrobicide (Padalia *et al.*, 2014) and also a rich source of lutein a natural pigment (Deineka *et al.*, 2008) that can give a good orange shade to foods. The status of marigold, as a source of natural colourant has been reviewed by Verghese (1996, 1998) and Sowbhagya *et al.*, (2004). Being rich source of lutein and other carotenoids, marigold flowers make an excellent source of valuable nutraceuticals and safe natural orange colorant in food applications (Sowbhagya *et al.*, 2004). In plant breeding, male sterility has been applied as an effective and economical means of pollination control and it place an important role in plant adaptation and evolution (Darwin 1877). Marigold has a terminal capitulum composed of hundreds of florets with two different types: ray (sterile) florets in the periphery, and disk (fertile) florets in the centre. The male sterility was confirmed to be a recessive genic trait and was reported that spontaneous homeotic conversion of floral organs was the underlying cause of the male sterility in marigold by He *et al.*, (2010).

Hybrid varieties of *T. erecta* or *T. erecta* X *T. patula* are being cultivated globally, but F<sub>1</sub> hybrid seeds are expensive and their supply is restricted from a few renowned companies only. Marigold male sterile lines are used for cross-breeding purposes (Zhang *et al.*, 2005) and serve to make the hybridization process relatively efficient and economic. Recently it has been reported in marigold that crossing between male sterile lines and inbred lines given hybrid combinations with higher ornamental values, with obvious heterosis over the male parent for most of the ornamental traits (Ai *et al.*, 2015). Fortunately, the existence of male sterility system in marigold has made it easy for the crossing programs in the production of F<sub>1</sub> hybrids. However, the GMS system has the limitation

that it is difficult to obtain a 100% male sterile population, making it necessary to manually remove the 50% fertile plants in order to prevent contamination of the F<sub>1</sub> hybrid progeny which is time and labor consuming and also difficult to remove prior flowering. This must be achieved prior to flowering and consumes significant time, labor and money (He *et al.*, 2009). However, molecular markers may be used to facilitate the detection of important traits, thereby permitting breeding programmes for elite cultivars to be concluded in a shorter time and in a more cost effective manner (Tanksley *et al.*, 1989). Thus, the identification of molecular markers that are tightly linked to the male sterility locus would permit the early and efficient identification of individual plant genotypes within the breeding populations. There is a very recent report of AFLP and SCAR-based linkage map by He *et al.*, (2010) where they have reported that SCAR 4 marker has been linked to male sterility in marigold which is placed at a distance of 0.3 cM distance from the sterility locus on the linkage map showing that it is tightly linked to sterility gene.

Hence in the present study, to assess the efficiency and practical applicability of SCAR 4 linked marker for Marker Assisted Selection breeding programme it was attempted to validate linked marker in different genetic background of F<sub>2</sub> population and in 12 different apetalloid male sterile lines which confirmed its linkage with sterility gene.

## **Materials and Methods**

### **Primer**

The previously identified SCAR 4 marker which was reported to be linked to male sterility by He *et al.*, (2009) was validated in the present study. The sequences of SCAR4 marker used in the present study are as follows:

F1: TGAGTCCAAACCGGACCCGG; R1:  
ACTGCGTACGAATTAGCACACATTA;

### **Plant material**

An F<sub>2</sub> segregating population of 226 progeny plants were generated following the crossing programme involving a cross between contrasting parent's male sterile line (IIHR10521AB) and a pure line (IIHRMY7). Finally, a total of 226 F<sub>2</sub> plants segregating for sterility character were obtained which were found to be morphologically indistinguishable prior to flowering. After flowering, the phenotyping of F<sub>2</sub> progeny was done based on floral traits. The flowers of the male fertile plants had normal petals in the ray and disc florets whereas, the petals of the ray and disc florets of the male sterile plants degenerated or developed as white filament-like petals and the stamen became yellow filament without pollen inside (Plate 1).

The other different genetic background such as 12 different Apetaloid male sterile lines (Table 1) was also used in the present study to validate the linked marker.

### **DNA extraction**

The samples for DNA extraction were collected separately from young leaves from both the parents used in crossing programme and from each plant of the 226 F<sub>2</sub> plants at young seedling stage using CTAB method of DNA extraction as described by Doyle and Doyle (1990) with some minor modifications including phenol treatment and the final DNA concentration were adjusted to 60 ng for PCR analysis.

### **Bulk Segregant Analysis (BSA)**

For bulked segregant analysis (BSA) (Michelmore *et al.*, 1991), equal amounts of DNA were taken from 10 fertile plants and 10

sterile plants randomly from F<sub>2</sub> progeny and were pooled to create 'F bulk' (BF) and 'S bulk' (BS), respectively. Initially the two bulk samples were subjected to analysis using SCAR marker to confirm polymorphism between parents and bulk samples to avoid the laborious screening of whole mapping population. Later, it was used for further screening of whole mapping population to identify the linkage with sterility.

PCR amplification was achieved in a Eppendorf Thermocycler (Eppendorf mastercycler Germany) programmed for initial denaturation at 94° C for 2 minutes, followed by 35 cycles; each cycle consisting of denaturation at 94° C for 45 seconds, primer annealing at 60° C for 45 seconds and primer extension at 72° C for 45 seconds and a final extension for 10 minutes at 72° C and hold at 12 ° C. The PCR reaction was carried out in a final volume of 20 µl reaction mixture containing 60 ng of template DNA, 0.2 X buffer, 0.2 mM dNTPs, 0.5 mM of MgCl<sub>2</sub>, 0.3 U of Taq DNA polymerase, 0.025 pM each of forward and reverse primer.

### **Genotyping of F<sub>2</sub> population**

An F<sub>2</sub> segregation population size of 226 individual plants were screened using the SCAR 4 primer which showed polymorphism between BF and BS and SCAR profiles were generated using PCR and visualized on 2% Agarose gel and analyzed for further analysis.

### **Analysis of SCAR profiles**

Amplification profiles obtained by SCAR 4 primer for parents and all F<sub>2</sub> plants were estimated by comparing DNA fragment sizes on agarose gel with 100 bp DNA size markers. The bands were scored as 'A' for the presence of band size same as female parent/seed parent *i.e.*, sterile parent, scored as 'B' for the presence of band size same as male

parent/pollen parent i.e., fertile parent and scored as 'H' for the presence of both the male sterile and fertile parent bands in case of heterozygous F<sub>2</sub> plants.

## **Results and Discussion**

### **Screening of SCAR 4 marker linked to the sterility gene**

Initially the marker was screened for polymorphism between the parents used in the present study like male sterile line (IIHR10521AB) and a pure line (IIHRMY7). The analysis profile generated by SCAR 4 marker revealed a clear polymorphism between parents with same band size as reported previously. The primer produced amplification product size of 520 bp in sterile parent and 330 bp size in fertile parent.

### **Bulk Segregant Analysis**

Later, for bulked segregant analysis, the primer was used to screen for polymorphism between the Sterile bulk (SB) and Fertile bulk (FB) populations including parents again. The amplification profiles revealed a clear polymorphism between bulk populations with same amplicon size as in parent. To verify the implied linkage of this marker with male sterility locus, twenty individuals from the 'F' and 'S' bulks were examined individually in more detail with respect to the polymorphic bands. The results of BSA with similar band pattern as in parents and bulks revealed that it may be linked to sterility (Plate 2). Subsequently, all the 226 F<sub>2</sub> progeny were screened with the SCAR 4 marker to assess the primary linkage to the sterility locus.

### **Screening of F<sub>2</sub>**

The segregating F<sub>2</sub> population of 226 plants, derived from a cross between Male sterile (IIHR10521AB) and inbred line (IIHRMY 7),

comprised 164 male fertile and 62 male sterile plants (determined by flower morphology). Thus, the population displayed a 3: 1 ratio of male fertile (mf) to male sterile (ms) plants. This was consistent with the segregation of a recessive gene in the F<sub>2</sub> population, and which was designated as *Tems*, a recessive gene controlling the male sterility in marigold.

Based on the analysis of profiles obtained by genotyping of F<sub>2</sub> population obtained using SCAR 4 marker, it was clearly showed that the marker segregated very clearly and consistently fitting the mendalian ratio of 3:1 ratio except for few samples showing recombination when screened among 226 F<sub>2</sub> populations showing that the marker is linked to male sterility in marigold (Plate 3). The primer SCAR 4 produced a band size of 520 bp in Sterile parent and 330 bp in Fertile parent and similar banding pattern was observed in sterile plants and fertile plants of F<sub>2</sub> progeny and both the bands were present in case of heterozygous progeny (hybrids). The strong correlation of these polymorphic bands with the male sterility/fertility trait established that this SCAR 4 is linked very closely to the sterility gene and hence can be efficiently used for selection of parents in marker assisted breeding selection programmes.

### **Screening and validation of SCAR 4 among different apetalloid sterile lines**

To further validate and confirm the linkage of SCAR 4 marker which was found to be linked to sterility gene locus via linkage map construction in the previous study, SCAR 4 was further screened among 12 different apetalloid male sterile lines (Table 1). The results of amplification profiles confidently revealed the linkage of SCAR 4 with the sterility gene locus in marigold by showing the similar banding pattern and polymorphism like in parents among different apetalloid sterile lines used (Plate 4).

In any plant breeding and crop improvement programme of any crop species, the existence of male sterility play a vital role, since male sterility has been applied as an effective and economical means of pollination control in breeding programmes. Fortunately, the existence of male sterility in Marigold has blessed this ornamentally important crop in the development of commercially important hybrids. Male sterility mediated F<sub>1</sub> hybrids are gaining more vogue and becoming profit oriented in many ornamental crops and also in Marigold. Identifying a molecular marker tightly linked to male sterility would permit the prior and planned manner of differentiating the sterile and fertile plants thereby concluding the breeding programme in a short time and in a very cost effective manner. After identification validating the linked marker is very much essential to assess the practical utility of linked marker as a step towards marker assisted breeding selection programme. Hence in the present, we have validated the previously reported SCAR marker linked to genetic male sterility in marigold for confirming its efficient utilization in MAS programme.

### **Bulk Segregant Analysis (BSA)**

Based on the results of screening for polymorphism, SCAR 4 marker generated polymorphism between sterile and fertile parents and was selected for further screening of F<sub>2</sub> mapping population. To avoid the laborious screening of a large number of F<sub>2</sub> plants for identifying the polymorphism of marker, we initially used Bulk segregant analysis strategy (Michelmore *et al.*, 1991) to identify the linkage of marker by screening SCAR 4 marker among SP (Sterile Parent), FP (Fertile Parent), SB (Sterile Bulk) and FB (Fertile Bulk). Based on the results, the marker produced polymorphic bands between parents, SB and FB by showing clear polymorphic band size. Similarly, the BSA has been widely used in various crops by Hui

*et al.*, (2019); Thakur *et al.*, (2014); Ragina and Sadhankumar (2013); Yanping *et al.*, (2013); He *et al.*, (2009) and Bi-Hao *et al.*, (2009) for detecting markers linked to genes conferring particular character and is a powerful method for identifying molecular markers that show association with gene of interest or a specific region of the genome (Ren *et al.*, 2012, Salinas *et al.*, 2013).

### **Screening F<sub>2</sub> population and apetalloid sterile lines**

To identify the markers linked to the *Tems* gene, SCAR 4 marker which showed polymorphism between two bulks, FB and SB in Bulk Segregant Analysis was screened among whole 226 F<sub>2</sub> populations for further analysis of genetic distances and to reveal the preliminary linkage of marker to sterility. The results of genotyping revealed that the marker segregated consistently in 3:1 ratios for fertility and sterility with respect to the amplification of specific amplicons, except for few samples showing recombination or segregation distortion when screened among 226 F<sub>2</sub> populations showing that it is tightly linked to male sterility in marigold. The segregation distortion is widespread in plant populations and is a common feature of plant genetic linkage maps. Both biological factors and technical problems potentially contribute to segregation distortion (Yanping *et al.*, 2013). Also the results of screening of linked marker among twelve different apetalloid sterile lines revealed and confirmed the potential application of SCAR 4 marker in MAS breeding programmes for accurate selection of different lines by producing the same banding pattern as parents among different apetalloid sterile lines. This again validated the tight linkage of SCAR 4 marker with sterility gene locus in marigold thereby offering a powerful tool for accurate selection of parental lines at their early seedling stages itself through marker assisted selection (MAS).

**Table.1** List of apetalloid male sterile lines used for validation of linked markers

Sl. No	Apetalloid lines	Validation
1	IIHRMOS-1	Validated
2	IIHRMOS-14	Validated
3	IIHRMOS-15	Validated
4	IIHRMOS-17	Validated
5	IIHRMOS-19	Validated
6	IIHRMOS-11	Validated
7	IIHRMOS-23	Validated
8	IIHRMOS-24	Validated
9	IIHRMOS-66	Validated
10	IIHRMOS-73	Validated
11	IIHRMOS-31	Validated
12	IIHR10521AB	Validated

**Plate.1** Parents used for development F2 Mapping population

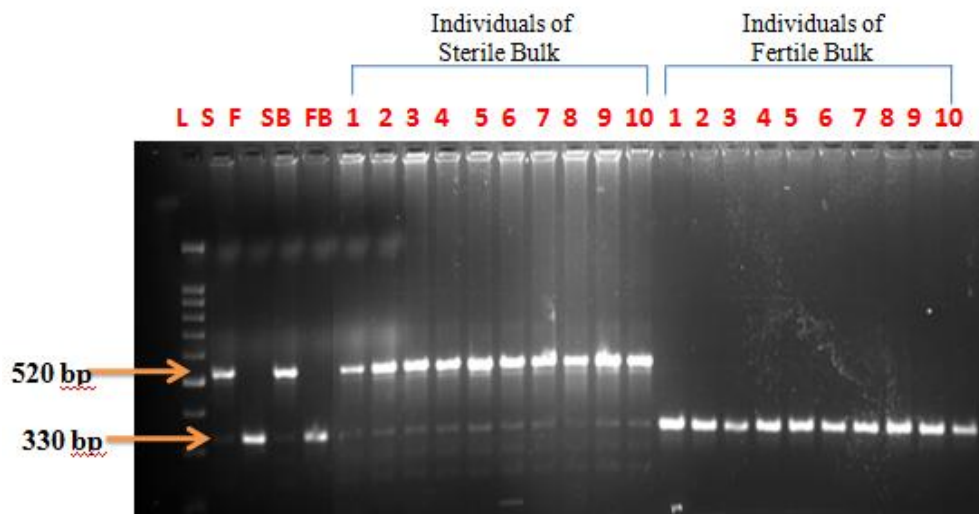


Mate Parent (Inbred line IIHRMY7)

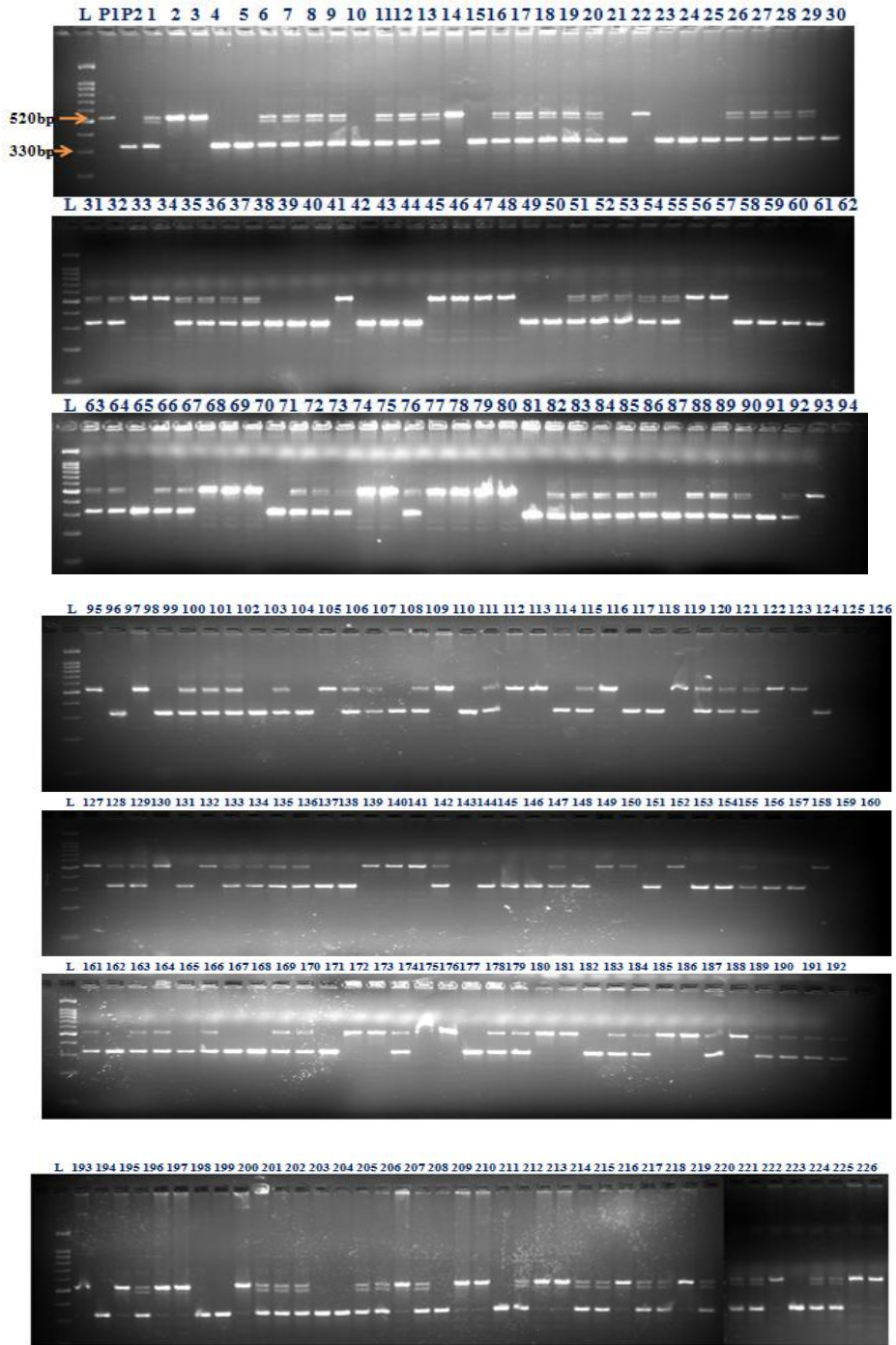


Female Parent (Apetaloid Sterile line IIHR10521AB)

**Plate.2** The PCR amplification of SCAR 4 marker in parents, Sterile Bulk (SB), Fertile Bulk (FB), Individuals of sterile and fertile bulks

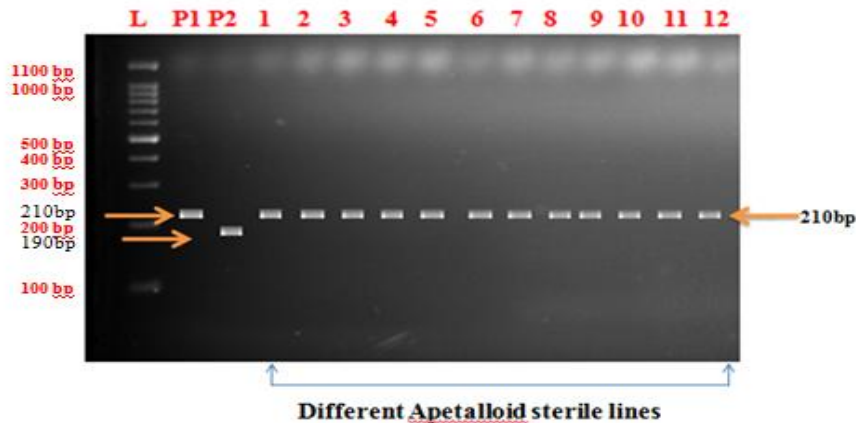


**Plate.3** Genotyping of Parents and F<sub>2</sub> progeny using SCAR 4 primer



L- 1000 bp Ladder; P1- Sterile parent; P2- Fertile parent; 1-226 indicates the progeny of F<sub>2</sub>

**Plate.4** Validation of sterility linked marker SCAR 4 in 12 different Apetalloid male sterile lines



The results showed that how best this linked marker could be used as a selection marker in MAS breeding that facilitate narrowing down the population with fertile lines, retaining only sterile progenies during hybridization programme in marigold while developing outstanding hybrids. Similarly the markers identified to be linked to various traits in different crops have been validated for their use in MAS by Yagi *et al.*, (2014) in Carnation (SSR marker linked to double flower type); Bhati *et al.*, (2018) validated SSRs markers linked to *Rf* genes in diverse rice breeding lines. SCAR marker, SCU176-534 linked to bacterial wilt resistant in tomato was validated for MAS by Kumar *et al.*, (2018). SSR marker AVRDC-PP12 linked to the male sterility gene *ms10* in chilli was validated by Aulakh *et al.*, (2017). SSR markers-linked to *Rf* locus in Rice were validated using eight tester lines by Raghavendra and Hittalmani (2015). Dhanya *et al.*, (2014) validated the marker orf 725 linked to onion male sterility by screening different onion male sterile, maintainer and F<sub>1</sub> hybrids which resulted in the confirmation of identified molecular marker orf 725 is capable of distinguishing male sterile and maintainer genotypes. SCAR marker syau-scr04 linked to male sterility gene in Chinese cabbage, was applied for MAS by Hui *et al.*, (2011) based

on which the new male sterile line GMS4 with 100 % male sterility and 100 % male sterile plants was bred successfully and the linked SCAR marker syau-scr04 accurately determined the plant genotypes showing that it could be efficiently applied for marker-assisted selection of the genetic male sterile line in *Brassica spp.* The two markers linked to sterility in welsh onion were confirmed by Gai *et al.*, (2010) using seven welsh onion cultivars which accelerated the selection of sterile and maintainer lines in welsh onion breeding via MAS. The SSR (GBM1267) marker linked to male sterility in Barley was validated by Emebiri (2010). The SCAR marker (syau\_scr01) and SSR marker (syau\_m13) linked to male sterility in Chinese cabbage were verified using BC4 and BC5 populations which proved their application in marker assisted selection breeding (Hui *et al.*, 2010).

In conclusion, once molecular markers have been linked to a trait of interest, these markers can be used to select desired lines from a large-scale population through marker-assisted selection (MAS), which saves both costs and time. Hence, the validation of previously identified linked marker (SCAR 4 linked to sterility gene) in different genetic background of F<sub>2</sub> and among different



apetaloid male sterile lines in our study proved the potential application of identified linked marker (SCAR 4) which ultimately contribute for marker assisted selection breeding programme in marigold. MAS enable accurate selection regardless of environmental factors and it had been applied in breeding of various crops (Debener 2001), but to the best of our survey, the development of a commercial marigold cultivar through MAS has not been reported. Therefore, the demonstration on the validation of linked marker in the present study represents the use of SCAR 4 in MAS which showed an important advance in the breeding of marigold. These results serve as a valuable resource for genetic research in Marigold mainly in MAS.

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