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Original Research Paper

Validation of Molecular Markers Genetically Linked to *S*-Cytoplasm and Restoration-of-fertility (*Rf*) Loci in Hot Pepper (*Capsicum annuum L.*)

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ABSTRACT

Existence of CGMS system in hot pepper is due to the rearrangements in the mitochondrial genome and is largely used in economized and pure F_1 hybrid seed production around the world. The *orf456*, a new ORF present at flanking region of the *coxII* gene at the 3' end, was distinguished male sterile cytoplasm in hot peppers along with *atp6-2*gene. In the current study, eighteen pepper genotypes (nine each of A and corresponding B lines) of varied origin were used to validate with two male sterile cytoplasm (*S*-cytoplasm) specific sequence characterised amplified region (SCAR) markers *viz*., *atp6-2*$_{(875\ bp)}$ and *orf456*$_{(456\ bp)}$ and one restoration-of-fertility (*Rf*) locus specific marker, CRF$_{(550\ bp)}$. The results clearly showed that the presence of CMS-*S*-cytoplasm and absence of restoration-of-fertility (*Rf*) gene in the pepper genotypes studied and is comparable with the phenotypic data. In view of the outcomes it has been reasoned that the accessible *S* and *Rf* markers available in the public domain are reproducible and can be promptly utilized for marker assisted selection (MAS) in hot pepper crop improvement program.

Keywords: CGMS, Hot pepper, Marker Assisted Selection, Mitochondria, ORF.

INTRODUCTION

Peppers are commercially grown as a spice and vegetable crop. Hot pepper is a Solanaceous crop, originated in Central and South America, and is introduced to India over 500 years ago.Among the domesticated species, *Capsicum annuum* L is one of the most extensively cultivated pepper species in India. In India, 75% of chilli production is from the southern states *viz*., Andhra Pradesh, Telangana, Karnataka, Tamil Nadu and Maharashtra. Concerted efforts in thecrop improvement program in pepper resulted in release of many improved varieties and F_1 hybrids for commercial cultivation. Utilization of male sterile systems in F_1 hybrid seed production of peppers is exceptionally economical.

Male sterility in crops is due to a failure to produce functional pollen or anthers (Grelon*et al.,* 1994, Pruitt and Hanson, 1991; Budar and Pelletier, 1994). CMS/ CGMS is exploited for the development of F1 hybrids in many crops around the world (Hanson, 1991; Hanson and Bentolia, 2004; Miller and Bruns, 2016). Generally,CMS resulted due to the rearrangements in the mitochondrial genome sequences,which in turn results in the arrangement ofnew open reading frames (ORF) which alter the expression of normal genes of the mitochondrial ATP synthesis complex (Pruitt and Hanson,1991; Budar and Pelletier, 1994). The rearrangements within the sub unit genes of ATP synthesis, such as atp 4, 6, 8 and 9 (Pruitt and Hanson, 1991; Hanson and Bentolia, 2004; Schanable and Wise, 1998 Pruitt and Hanson, 1989) are responsible for the CMS in crops and other gene rearrangements observed in pepper lines will be contributed by *coxII* and *nad9*.

Hot pepper genotype PI164835, a collection from India was the first CMS line reported (Peterson, 1958), and is being used in production of F_1 hybrid seeds all over the world (Reddy *et al.,* 2002). In this CMS line, a new ORF viz., *orf456* was found as flanking region of the *coxII* gene at the 3' end. The *atp6-2* gene is believed to be regulated through restoration-of-fertility (*Rf*) loci at the transcriptional level and the *orf456* is regulated at post transcriptional or translational level

Table 1. Molecular markers used for the validation of male sterile lines in the present study

Marker name (Nature)	Primer Sequence (52 to 32)	Annealing temperature (^{O}C)	Expected amplicon size of primer (bp)	Reference
atp6-2 (SCAR)	F AGTCCACTTGAACAATTTGAAATAATC R - GTTCCGTACTTTACTTACGAGC	58	875 bp	Ji *et al.* (2013)
orf456 (SCAR)	F - ATGCCCAAAAGTCCCATGTA R - TTACTCGGTTGCTCCATTGTTT	60	456 bp	Kim *et al.* (2007)
CRF (SCAR)	F - GTACACACCACTCG-TCGCTCCT R - TTCTTGGGTCCCTTT-CTTCCAA	55	870 bp	Gulyas *et al.* (2006)

(Kim *et al.,* 2006; Kim *et al.,* 2007), are responsible for CMS trait.In the present study, the four stable CGMS lines developed and being use dinpepper improvement program at ICAR-IIHR, Bangalore are validated with the two male sterile cytoplasm (*S*-cytoplasm) trait linked markers, *atp6-2* and *orf456* and one restoration-of-fertility (*Rf*) loci linked to *CRF* marker.

MATERIALS AND METHOD

Plant material

An aggregate of nine male sterile and their comparing nine maintainer lines were utilized in the current study are referenced in the Table 2.

Phenotypic evaluation of male sterility

The phenotypic evaluation of male sterility and fertility in lines were carried out by the visual observation at the flowering stage.The male sterile plants showed no pollen grains with shriveled anther lobes, whereas the male fertile plants have bulged anther lobes with abundant pollen grains (Plate 1).

Pollen morphologyandsize

The freshly unopened flower samples of male sterile and male fertile plants were gathered from the field in the early dawn,and put away in impenetrable zip lock polythene covers over the ice package to keep up the freshness. The pollen grains were collected from the dehisced anther lobes independently from individual flowers, frozen on the liquid nitrogen and stored them at -195^{0} for further studies. For morphological examinations, the individual pollen grains were directly dusted on to the slides and length and breadth of the individual grains were measured using scanning electronmicroscope (TM3000, Hitachi, Japan). The reproductive parts of both male sterile and male fertile flowers and the cross section of the anther lobes were additionally seen under the scanning electronmicroscope (TM3000, Hitachi, Japan), in order to study the morphological difference between the male sterile and male fertile flowers.The stereo microscopy images of the dehisced flowers were additionally examined (ZEISS Stereo zoom microscope Stemi 508 doc, Germany).

DNA extraction:

The total genomic DNA was isolated from the leaves of one month old seedling using 4% CTAB plant extraction protocol (Doyle and Doyle, 1990). The genomic DNA samples were qualitatively checked in 0.8% agarose gel and quantitatively by using UV-spectrophotometer. The concentrated DNA was diluted to 20ng/µL according to the spectrophotometer reading and thus diluted DNA is used as the template in PCR for genotyping with specific molecular markers.

PCR conditions and validation of molecular markers:

The polymerase chain reaction master mixture contained 2µL of 10X buffer, 2µL 25 mM $MgCl_2$, 2.5µL 1mM dNTP, (3b Blackbio, Spain) 1.5µL of 10µM of forward and reverse primer, 0.5µL 1U *Taq* DNAPolymerase (3b Blackbio, Spain) and 2µL of 20ng template DNA. The PCR conditions for the validation of the three SCAR markers were carried out as mentioned here. Initial denaturation at 95^{0} for 5 minutes accompanied with 30 repeated cycles of denaturation at 94^{0} for 60 seconds, annealing as given in the Table 1 for 60 seconds, extension at 72^{0} for 60 seconds and final extension at 72^{0} for 5 minutes. The reactions were carried out in the thermocycler (Eppendorf, Germany). PCR amplified fragments were separated on 1.5% agarose gel/1X TBE (w/vol), stained with ethidium bromide dye and

Table 2. Results of the markers screened for CGMS lines

Sl.No.	Sample Name	PCR Amplification of SCAR markers			Observed Phenotype	Expected Genotype
		atp6-2	*orf 456*	*CRF*		
Male sterile lines						
1	IIHR 3285 A	+	+	-	Sterile	S
2	IIHR 3226 A	+	+	-	Sterile	S
3	IIHR 3287 A	+	+	-	Sterile	S
4	IIHR 3228 A	+	+	-	Sterile	S
5	IIHR 4560 A	+	+	-	Sterile	S
6	IIHR 4561 A	+	+	-	Sterile	S
7	IIHR 4558 A	+	+	-	Sterile	S
8	IIHR 4553 A	+	+	-	Sterile	S
9	IIHR 4555 A	+	+	-	Sterile	S
Male fertile lines						
10	IIHR 3285 B	-	-	-	Fertile	N
11	IIHR 3226 B	-	-	-	Fertile	N
12	IIHR 3287 B	-	-	-	Fertile	N
13	IIHR 3228 B	-	-	-	Fertile	N
14	IIHR 4560 B	-	-	-	Fertile	N
15	IIHR 4561 B	-	-	-	Fertile	N
16	IIHR 4552 B	-	-	-	Fertile	N
17	IIHR 4554 B	-	-	-	Fertile	N
18	IIHR 4556 B	-	-	-	Fertile	N
19	Control R- line	-	-	+	Fertile	N

(+) amplification; (-) non-amplification

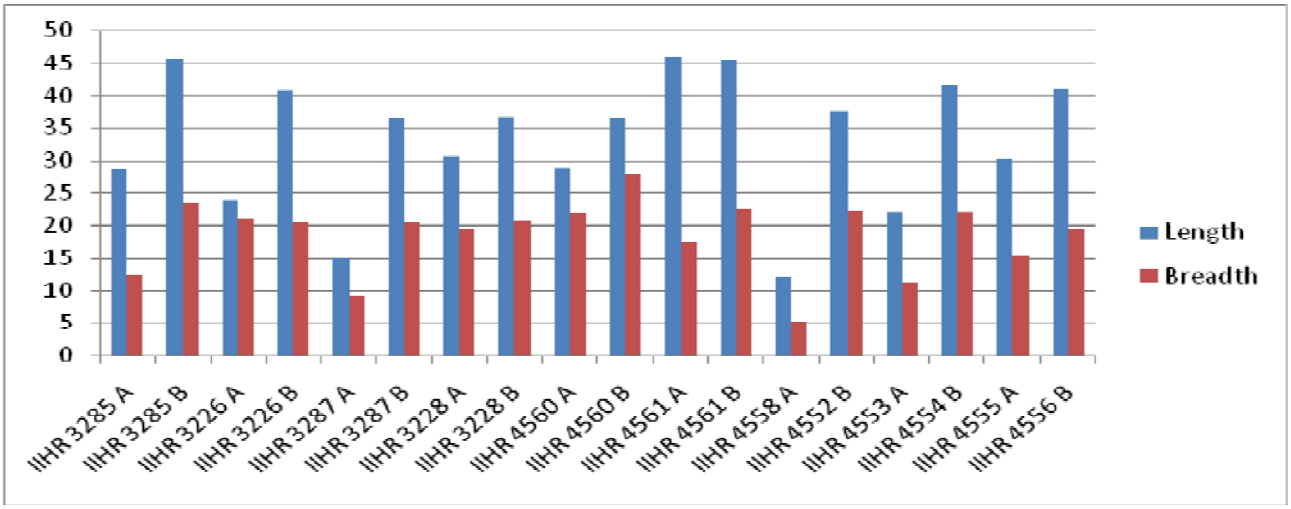


Fig 1. Bar diagram showing the measurement of individual pollen grains size in male sterile vs male fertile flowers using scanning electron microscope

documented under neath the ultra violet light (UVI Pro Platinum, Cambridge, U.K). The experiments were repeated for three consecutive times with each marker for confirmation of results.

Cloning and sequencing:

The PCR amplified fragments of atp6-*2* gene in male sterile lines were separated on 1% agarose stained with EtBr gel, excised and purified the fragments using Nucleospin® Gel and PCR Clean-Up Kit (Macherey-Nagel, Germany). Five µL of the eluted product was ligated into pTZ57RT cloning vector system. The pTZ57RT vector containing the ligated DNA was successfully transformed into DH5α strain of *E.coli.* Transformed colonies were spread on Luria Bertani agar/Ampicillin/X-gal/IPTG plates and were identified through blue white screening after incubation at 37^0 overnight. Recombinant colonies were confirmed using colony PCR, further plasmid was isolated using alkaline lysis method. The isolated plasmids were confirmed for the presence of insert (*atp6-2 gene)* by digestion with the restriction enzyme, EcoRI and the restriction digested products were separated on 1% agarose/ EtBr gel to differentiate two distinct bands of vector and the 850bp insert respectively. Before sequencing, PCR product clean-up was performed using Nucleospin® Gel and PCR Clean-Up Kit (Macherey-Nagel, Germany). The sequencing was carried out in ABI-3710 Prison automated DNA analyzer (Europhins, India).

RESULTS AND DISCUSSION

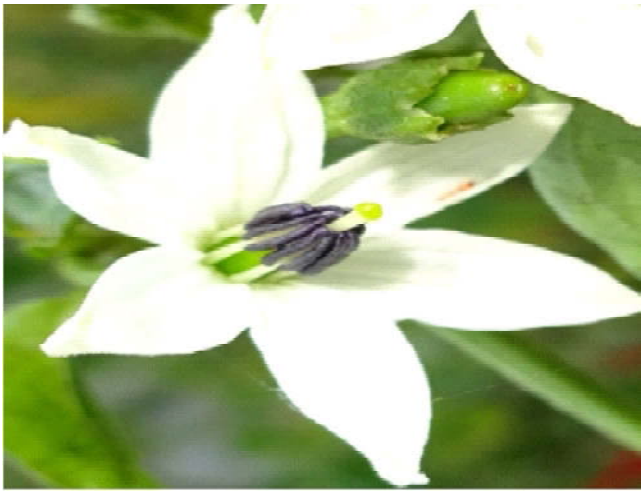
We used two male sterile cytoplasm (*S*-cytoplasm) trait linked markers,*atp6-2*$_{875}$and *orf456(*Ji *et al.,*2013and Kim *et al.,* 2005, 2007) and one restoration-of-fertility (*Rf*) loci linked marker *CRF* (Gulyas *et al.,*2006) to validate nine male sterile and their corresponding nine maintainer lines. CMS linked SCAR marker*orf456* amplified in allthe male sterile genotypes (*S*-cytoplasm), at an expected base pairs of 456 as shown in the Fig. 2 and this 456bp amplicon size was absent in all corresponding maintainer lines (*N*-cytoplasm) (Fig.2c, Table 2).Instead of amplifying at expected amplicon size of 875bp, *atp6-2* marker amplified at 850 bp in all the nine male sterile genotypes (*S*-cytoplasm) (Fig.2b, Table 2).In order to confirm the 25bp difference in the amplicon size, further cloning and sequencing was undertaken. Five clones each of the male sterile lines were selected, plasmid isolated, purified and further sequenced (ABI-3710 Prisom automated DNA analyzer). Sequence obtained from ABI-3710 Prisom automated DNA analyzer was analysed from NCBI site (www.ncbi.nlm.nih.gov) and checked for nucleotide sequence identity of the observed sequences and found that there is almost 99% identity for *Capsicum annuum atp6-2* subunit.The presence of the expected amplicon pattern in all nine male sterile genotypes (*S*-cytoplasm) proved that the mitochondrial gene associated *atp6-2* subunit is responsible for the transcription of the *orf456* novel gene which indeed responsible for the cause of CMS in the cultivar varieties of hot peppers. Meanwhile, the nine corresponding male fertile/ maintainer lines (*N*-cytoplasm) failed to amplify at the expected amplicon size. The CMS lines which are phenotypically male sterile are genotypically carrying a sterile cytoplasm, *S* with *rfrf* loci and all the maintainer or fertile B lines are genotypically carrying a normal cytoplasm, N with *rfrf* loci.The one *CRF-SCAR* marker specific to restoration-of-fertility (*Rf*) locus as expected failed to amplify the 550bp fragment in any of the nine cytoplasmic male sterile (A) lines or cytoplasmic male fertile/maintainer (B) lines (Fig 2,Table 2).The complete absence of the *CRF-SCAR* marker in all genotypes used for the current study proves that these samples didn't carry a restoration-of-fertility*, Rf* loci, indicating that the cytoplasm looks genotypically normal, N or sterile, S. Even though there are markers for identification of restoration-of-fertility (*Rf*) in hot pepper (Kumar *et al.,* 2009, Kim *et al.*, 2005, Zhang *et al.,* 2000),*CRF-SCAR* marker (Gulyas *et al.,*2006) is the most commonly and widely used molecular marker for the detection of presence or the absence of restoration-of-fertility in CMS lines of hot pepper.

Further, using the scanning electron microscope (SEM) the morphological variation in pollen grain size among the nine male sterile and their corresponding maintainer lines (Fig.1& Plate 2) was studied measuring the length and breadth of pollen grains (Plate 2). Maximum variation in pollen grain length was observed among A lines compared to B lines, and it ranged from 12.2 to 46.2µM and 36.4 to 45.7µM, respectively. Similarly, maximum variation in pollen grain breadth was observed among A lines and ranged from 5.24 to 21.9 µM, whereas it ranged from 19.4

Plate 1. Images of male sterile *vs* male fertile flowers

IIHR3285 A

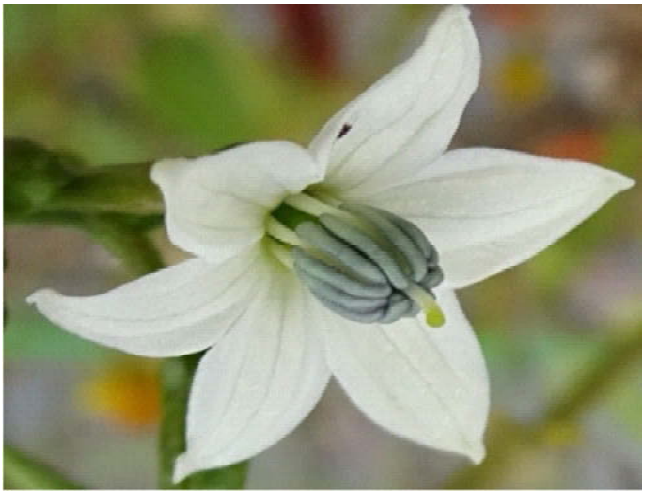
IIHR3285 B



IIHR3287 A

IIHR3287 B

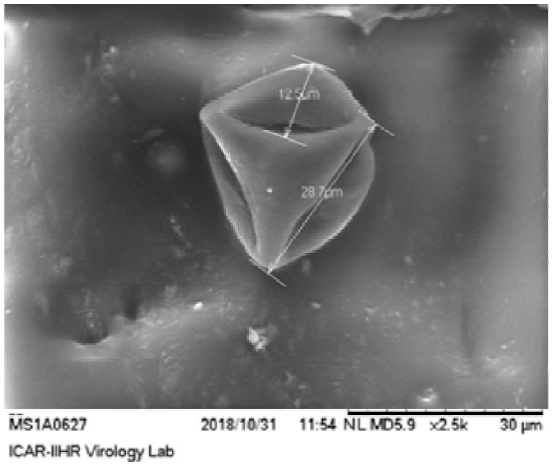
IIHR3228 A



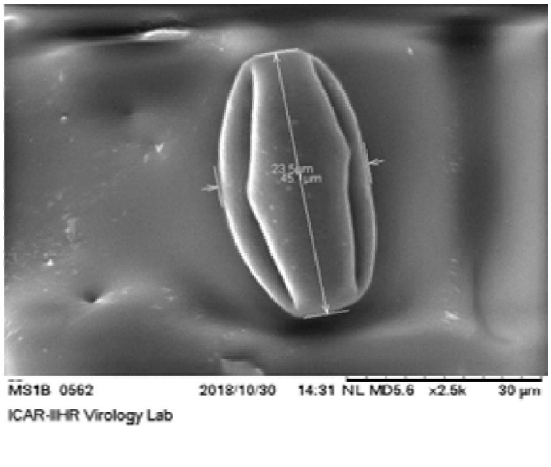
IIHR3228 B

Male sterile (A) lines showing shrinked anther **Maintainer (B) lines showing bulged anther**

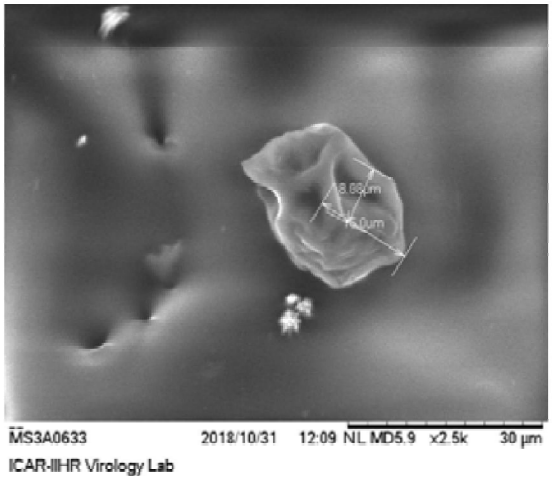
Plate 2. Images of male sterile *vs* male fertile anthers (Single pollen SEM images at 2.5k magnification)



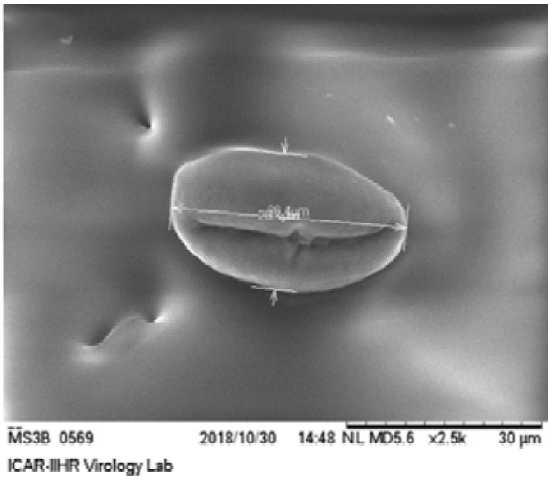
IIHR3285 A



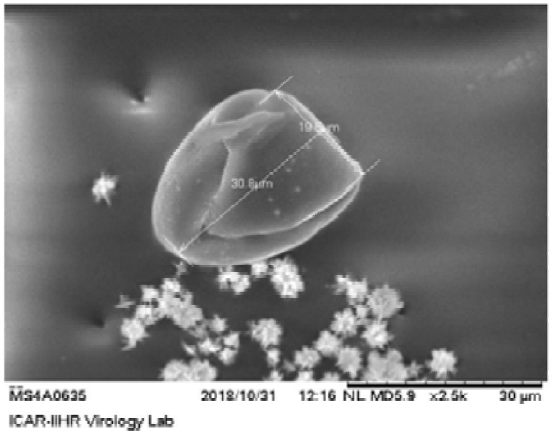
IIHR3285 B



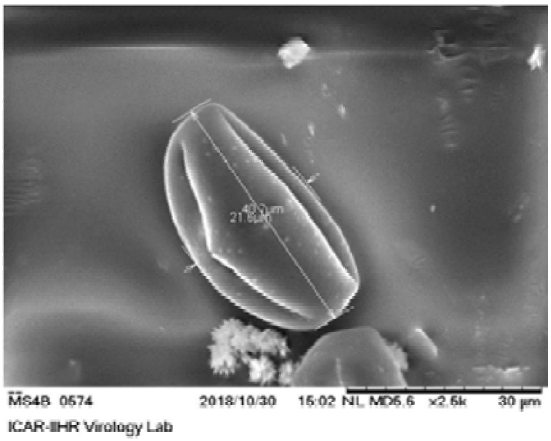
IIHR3287 A



IIHR3287 B



IIHR3228 A



IIHR3228 B

Male sterile (A) lines showing shrinked pollen grains

Maintainer (B) lines showing normal pollen grains

to 27.8µM among B lines (Fig. 1 & Plate 2), respectively.

The SEM images of the reproductive parts of the male sterile flowers morphologically found to be very shorter in size compared to the male fertile plants. The anther lobes of the male sterile flowers appeared to be shrivelled with less or shrunken pollen grains, whereas the male fertile plants have bulged anther lobes with abundant pollen grains (Plate 3b). So as to see the distribution of the pollen grains inside the anther lobe, the cross section of the anther lobe was studied. The SEM images clearly distinguished the male sterile plants had no visible pollens inside the tetrad pollen chambers, rather the male fertile plants produced numerous functional pollens (Plate 3c) attached to the tetrad anther chambers. The stereo

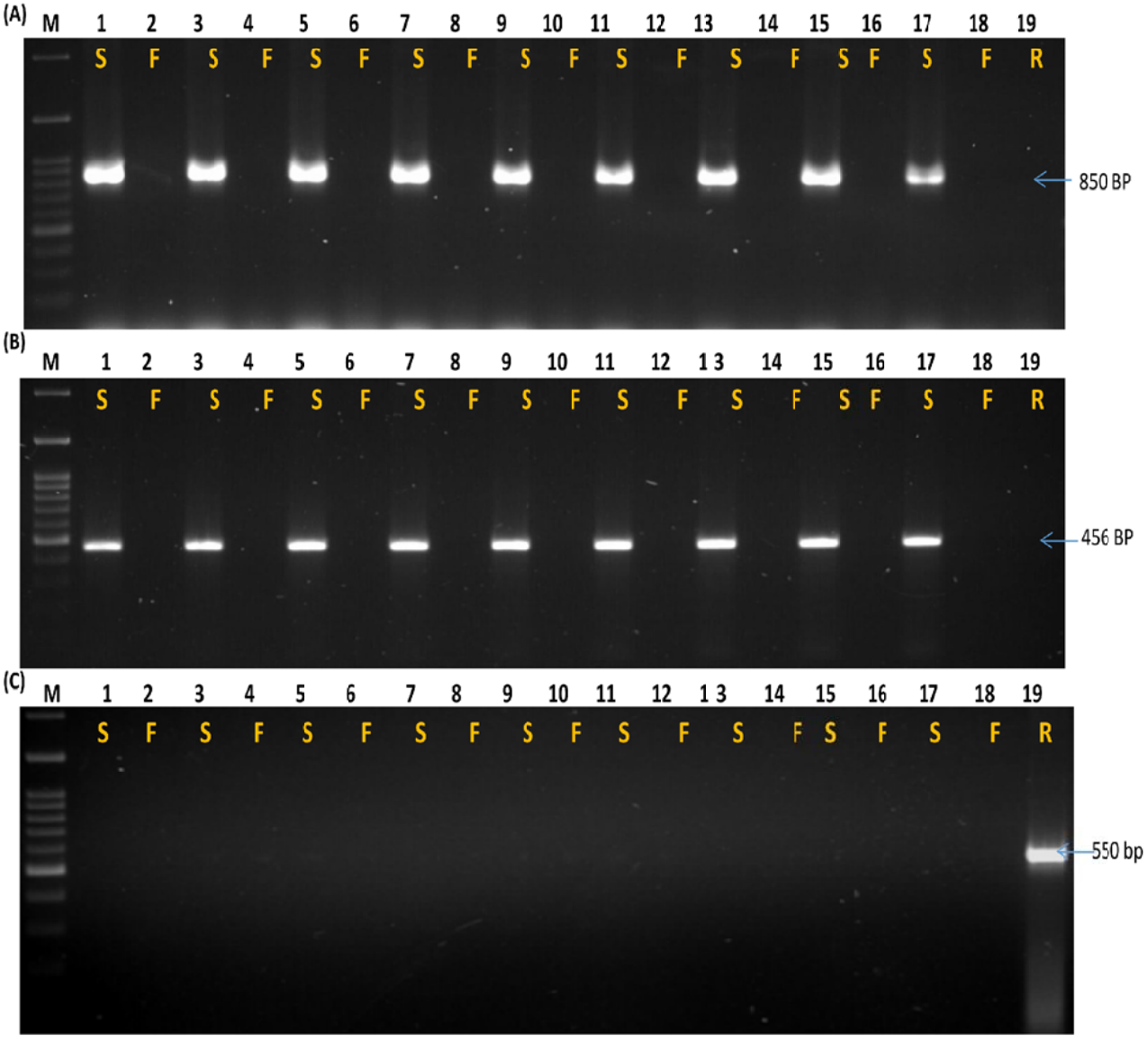
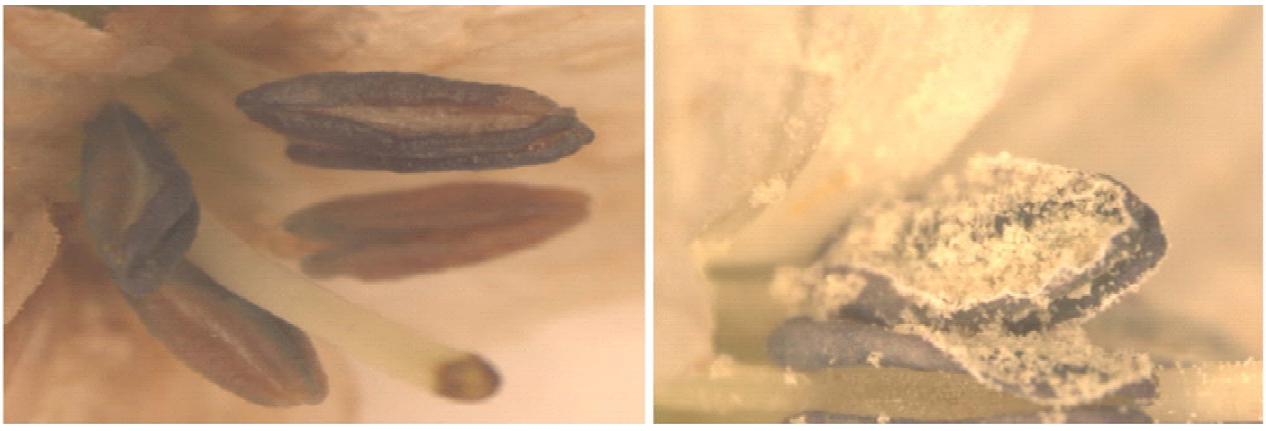


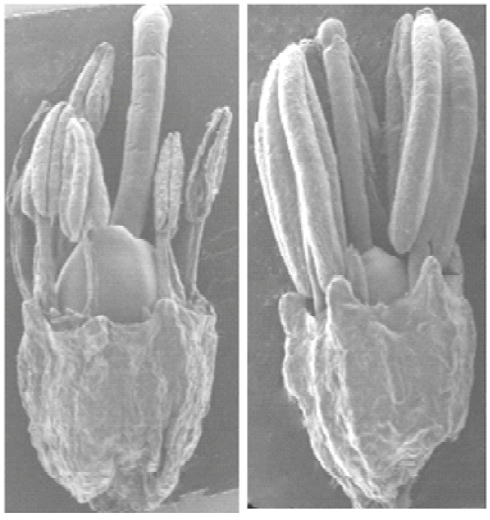
Fig 2: Gel picture showing the amplification results with the three molecular markers across eight pairs of sterile and fertile lines used (**A**) *atp6-2* marker (**B**) *orf 456* marker and (**C**) restorer of fertility gene specific *crf*marker. All PCR products were separated on 1.5% 1X TAE- agarose gel, stained with ethidium bromide dye. M= 100 bp ladder, serial number 1-18 indicates the sample order as given in the table no.2. S= male sterile line, F= male fertile line and R= restoral line. Arrow head indicates the band size obtained.

Plate 3. Male sterile *vs* male fertile (a) flower, (b) reproductive part and (c) cross section of anther lobe

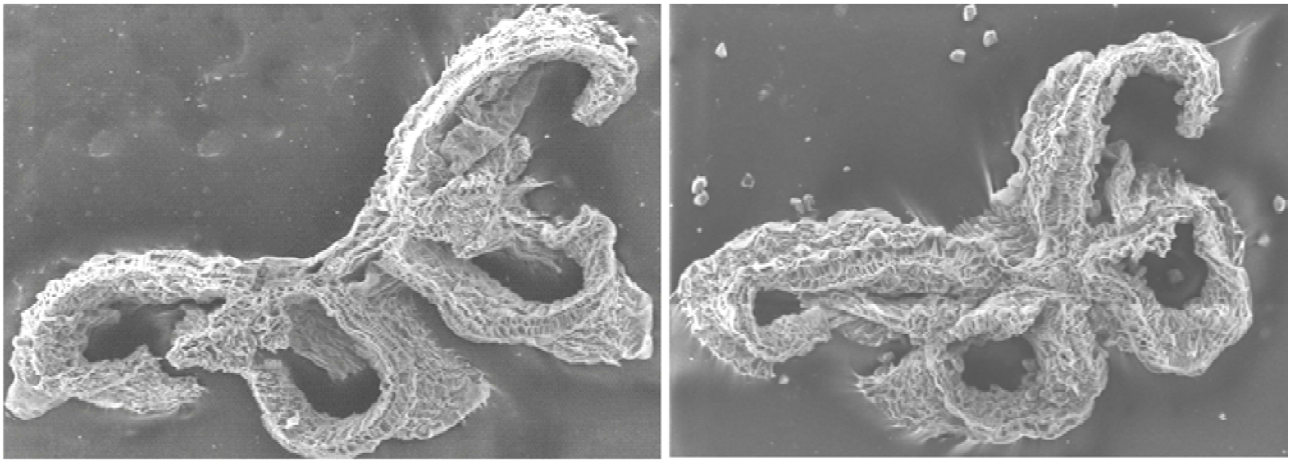
Male sterile **(a)** **Male fertile**



(b)



(c)



Male sterile (A) lines with no or very less pollen grains

Maintainer (B) lines with abundant pollen grains

microscopy images of the dehisced flowers clearly showed the absence of pollens at different magnification in male sterile plants where as presence of abundant pollen grains were visible in and out of the anther lobes of male fertile plants as shown on Plate 3a.

CONCLUSION

CMS in crops is caused due to a failure to produce functional pollen or anthers (Gómez 1999, Pruitt and Hanson, 1991). Previously, the two male sterile cytoplasm (*S*-cytoplasm) trait linked molecular markers viz., *atp6-2* and *orf456 (*Ji *et al.,*2013 and Kim *et al.,*2005,2007)were identified and characterised in CMS lines of hot pepper, were further used for the hybrid seed production in a commercial scale. The CMS pepper lines, were validated with the existing SCAR markers linked to the male sterility in pepper.The eight hot pepper lines namely IIHR 3285, IIHR 3226 , IIHR 3287, and IIHR 3228 (four CMS and 4 maintainer lines) developed at ICAR-IIHR, Bangalore and the other ten hot pepper lines (5 CMS and 5 maintainer lines) received from AVRDC, Taiwan, are having common sterile cytoplasm and restoration-of-fertility genes as were successfully validated using the three already known SCAR markers i.e., two male sterile cytoplasm (S-cytoplasm) trait linked to *atp6-2* and *orf456 (*Ji *et al.,*2013 and Kim *et al.,* 2005, 2007) and one restoration-of-fertility (*Rf*) loci linked marker *CRF* (Gulyas*et al.,*2006) and these molecular markers are highly reproducible at the genotypic level. Thus, these molecular markerscan be effectively used to recognize CMS from maintainer lines and fertility restorer lines and helps to fasten the breeding work to incorporate the CGMS system with varied fruit types and to incorporate disease resistant genes into A, B and R lines.

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