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Identification and tagging of fertility restorer (*Rf*) genes in chilli (*Capsicum annuum* L.) cultivars

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SUMMARY

A unique feature of CMS is that the expression of the trait is influenced by nuclear fertility restorer (*Rf*) genes .Gene specific primers were used in the present study to identify the accessions carrying the fertility restorer gene. Primers specific to *Rf* gene successfully amplified *Rf* region in G-4, S-49, Jwala, GVC-121, LCA-436, GVC-101, GVC-111, RHRC Pendent, Reshampatti cultivars indicating that these may be the potential restorer lines which can be utilized in heterosis breeding programme.

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Key words : *Capsicum annum*, CMS, *Rf* gene

Male sterility in pepper has been studied by Peterson (1958). Since then, much information dealing with the trait have been reported, including its isolation, mutagenic induction, inheritance, cytology and, particularly potential for hybrid seed production.

The CMS (cytoplasmic male sterile) phenotype is suggested to originate from some mutations in the mitochondrial genome of the male fertile progenitors as a result of some mutations of intra- or intermolecular recombination events. The association of CMS with abnormal mitochondrial gene expression has been reported in many plant species including maize, petunia, sunflower and common bean. Although it can appear spontaneously in nature, either in inter- or intraspecific crosses, for commercial hybrid seed production, often it is induced.

A unique feature of CMS is that the expression of the trait is influenced by nuclear fertility restorer (*Rf*) genes (Schnable and Wise, 1998). Nuclear restorer genes can suppress the effect of sterile cytoplasm and restore fertility to the next generation, which is a desirable requirement of the F1 commercial hybrid seed production. These fertility restorer genes are thought to block or compensate for cytoplasmic dysfunctions that are phenotypically expressed during pollen development. Although pepper is evaluated as a leading vegetable crop, the mechanism underlying CMS has not been sufficiently characterized yet. The mechanism by which the nuclear restorer gene acts to restore fertility is also poorly understood. Gene specific primers were used in the present study to identify the accessions carrying the fertility restorer gene.

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MATERIALS AND METHODS

Plant material

The experimental material used which consisted of thirteen cultivars of *Capsicum annuum* for determination of genetic diversity amongst the released cultivars. The cultivars and their origin are G-4 (Andhra Pradesh), AVNPC (Gujarat), S-49 (Gujarat), Jwala (Delhi), GVC-121(Gujarat), LCA-436(Andhra Pradesh), GVC-101(Gujarat), GVC-111(Gujarat), RHRC Pendent (Maharashtra), Punjab Gucchedar (Punjab), Kumthi (Local), Phule Jyoti (Maharashtra), Reshampatti(Gujarat).

Isolation of DNA from chilli leaves:

Genomic DNA was extracted from the leaves by Cetyl trimethyl ammonium bromide (CTAB) method (Zidani *et al.*, 2005) with some modifications. The young and healthy leaves from each cultivar of frozen tissue (0.300mg) was ground in a mortar and pestle in liquid nitrogen and homogenized in 1ml of preheated (600C) extraction buffer containing: 1M Tris-HCl (pH8.0); 1.4M Nacl; 20mM EDTA; CTAB 4% (w/v), 1% β-mercaptoethanol (added fresh in extraction buffer) and

10 µl proteinase k was quickly added to each microcentrifuge tube and vortexed to mix. The mixture was incubated 20min at 60^{0}C with occasionally mixing and genetic swirling. DNA was extracted twice with an equal volume of chloroform / isoamylalcohol (24/1; v/v) and precipitated with one volume of cold iso-propanol. The precipitated, washed with70% (v/v) ethanol for 5 min, dried in room temperature and resuspend autoclaved water. RNA was removed by digestion with RNAse A. The DNA was further purified using chloroform/isoamyl alcohol extraction and re-precipitated with ethanol and re-suspended in autoclaved water. The samples were stored at -20^{0}C.

PCR based assay for Rf gene tagging:

The presence of fertility restorer gene in *Capsicum annuum* varieties was checked using gene specific primers (Table 1). The primer used is given below.

Table 1 : Gene specific primer for Rf gene tagging in chilli (*C. annuum* L.)

Primer	Oligonucleotide primer sequence (5'……..... 3')	Reference
CRF3–S	ATTTTCAGATTGTGGCGACG	Gulyas *et al*., 2006
CRF1–S	CGACCATCACGACGAGG	

The reaction mixture for a 25µl reaction comprised 20ng template DNA, 15mM Mgcl2, 10mM dNTP's, Taq DNA polymerase (3U/µl), in 1x reaction buffer. Amplification by PCR was performed according to method given by Gulyas *et al.* (2006) with some minor modification included denaturation 5 min (94^0 C); then 33 cycles of 1 min at 94^{0}C; 1 min at 56.4^{0}C; and 1.30 min at 72^{0}C.

RESULTS AND DISCUSSION

In many CMS systems, male fertility can be restored by a series of fertility restorer (*Rf*) genes encoded in the nucleus. It has been proposed that the *Rf* genes block or compensate for mitochondrial dysfunctions that are phenotypically expressed during pollen development (Schnable and Wise, 1998). Thus, CMS-restorer systems appear to result from specific nuclear-mitochondrial interactions though little is known about the mechanism of fertility restoration in even the best-characterized systems. Molecular markers tightly linked to *Rf* loci have several applications in breeding programs. In many situations, geneticists do not know whether a new breeding line (or germplasm accession) should be classified as a B (Maintainer) or R (Restorer) line. To determine the status of these lines, the test cross of the lines to a male-sterile line has to be done and then scoring the resulting F_1 for male sterility/fertility. The identification of molecular markers tightly linked to *Rf* loci permits the classification of lines as either B or R without the need for test crosses. The transfer of fertility restoration gene(s) into different genetic backgrounds by repeated backcrossing is the most common and direct method for the development of new restorer lines. However, individuals with *Rf* genes need to be identified by crossing with the male-sterile line, and observing the resultant hybrids for male fertility restoration. This is a time-consuming and cumbersome process. With the development of molecular marker technology, it has been possible to identify molecular markers tightly linked to the fertility restoring (*Rf*) genes, and consequently selection can be conducted using these markers without the need for confirmation through phenotyping. Identifying and tagging fertility restorer genes (*Rf*) is a major step towards accomplishing this objective.

Previously, few molecular markers had been identified as being closely linked to the *Rf* gene. In the present study restorer gene specific primers (Yanagawa *et al*., 1996, Gene Bank Accessions: E15678 and E15679) were used to identify the restorer lines.

The PCR amplification results showed that the restorer gene specific markers could be distinguished in the fertile and CMS plants (Table 2). Upon PCR amplification, 9 out of 13 genotypes produced PCR fragments of size ranging from 893 to 950 bp specific to *Rf* gene, indicating that G-4, S-49, Jwala, GVC-121, LCA-436, GVC-101, GVC-111, RHRC Pendent, Reshampatti may be the potential restorer lines (Fig. 1). The amplified fragments specific for restorer gene were of slightly different molecular weights.

Table 2 : Details of conserved *Rf* gene analysis

Sr. No.	Cultivar	Presence/absence of *Rf* gene	Product size (bp)
1.	G-4	+	907
2.	AVNPC-131	-	-
3.	S-49	+	907
4.	Jwala	+	935
5.	GVC-121	+	921
6.	LCA-436	+	921
7.	GVC-101	+	921
8.	GVC-111	+	950
9.	RHRC Pendent	+	950
10.	Punjab Gucchedar	-	-
11.	Kumthi	-	-
12.	Phule Jyoti	-	-
13.	Reshampatti	+	893

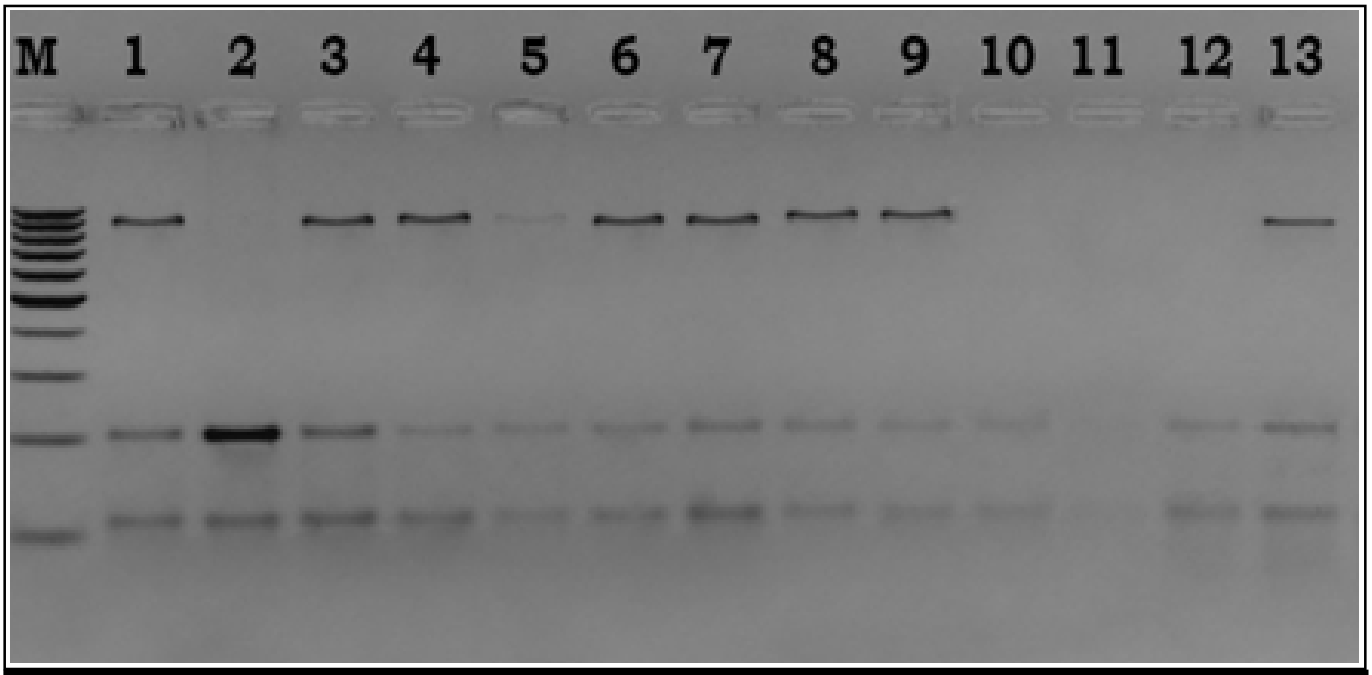

Fig. 1 : Amplification profile of *Capsicum annum* cultivars produced by primer specific *Rf* gene M-100bp,1. G-4 (Andhra Pradesh), 2. AVNPC (Gujarat),3. S-49 (Gujarat),4. Jwala (Delhi),5.GVC-121(Gujarat), 6. LCA-436(Andhra Pradesh),7. GVC-101(Gujarat),8. GVC-111(Gujarat), 9.RHRC Pendent(Maharashtra),10. Punjab Gucchedar (Punjab), 11. Kumthi (Local),12. Phule Jyoti (Maharashtra),13. Reshampatti(Gujarat)

The molecular weights of the amplified fragments were varying indicating the differences in the length of restorer gene, which may be due to spontaneous mutation.

The results obtained in these studies are in accordance with those obtained by Pakozdi *et al.* (2002). They used specific primers to reveal the molecular genetic differences between the CMS and restorer lines. An approximate 870bp fragment was amplified for all the fertile restorer plants, except the restorer line 207 which showed a fragment of 240 bp which may be due to the recombination nature of this particular line.

The validity of *Rf* gene associated RAPD markers in restorer and maintainer lines of pepper was studied by Kumar *et al*. (2004) showing potentiality of *Rf* gene markers in hybrid seed purity testing. These markers will be useful for development of new restorer lines by the transfer of the *Rf* gene into other breeding lines as well as for hybrid seed production. It can also be used for isolating the *Rf* gene by means of map based cloning.

To permit effective selection of target traits in plant breeding, molecular markers have been widely used as indirect selection tools. Some *Rf* gene-linked RAPD (Randomly amplified polymorphic DNA) (Zhang *et al*., 2000) and CAPS (Cleaved amplified polymorphic sequence) (Kim *et al*., 2006) markers have been reported, but the application of these markers for selection of diverse breeding lines and germplasms in practical pepper breeding programs has been limited, due to low reproducibility and the failure of PCR amplification in some breeding lines. The makers used in the present investigation gave reproducible results and thus can be used for fast and reliable identification of potential restorer lines.

Out of 13 genotypes, 9 produced PCR fragments of size ranging from 893 to 950 bp which were specific to *Rf* gene, indicating that G-4, S-49, Jwala, GVC-121, LCA-436, GVC-101, GVC-111, RHRC Pendent, Reshampatti may be the potential restorer lines.

Conclusion:

The *Rf* gene targeted for marker assisted selection (MAS) in the present study was a strong one. With the tagging of *Rf* using appropriate molecular markers, the efficiency of MAS for restorer gene can be further improved. It should then be possible to routinely use molecular markers to select restorers which can be then test crossed with appropriate CMS lines to confirm their fertility restoration and assess the magnitude of heterosis. The advent and application of molecular analysis of population genetic structures provides extremely useful information that will undoubtedly prove invaluable to future decision processes involved in the management and preservation of germplasm and genetic resources.

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