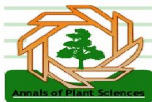

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# Validation of Molecular Markers Linked to Sterility and Fertility Restorer Genes in *Brassica juncea* (Linn) Czern and Coss.

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**Abstract:** The cytoplasmic male sterile (mori) *Brassica juncea* carries unaltered mt genome of *Moricandia arvensis*, and the fertility restoration is gamatophytic, governed by a single nuclear gene. This fertility restorer gene locus is unique in that, it is capable of restoring male fertility in two other alloplasmic CMS systems of *Brassica juncea*. The present study validated the application of a linked marker for identifying the restorers in segregating populations, which can accelerate breeding of restorer lines and thus, enhance efficiency of hybrid breeding in *Brassica juncea*. Presence of the CMS-associated orf genes was detected only in the CMS line, while SCAR primers generated amplicons in the fertile plants. This marker can be used to test the purity of hybrids as an effective substitute to the time consuming and laborious grow-out test (GOT).

**Keywords:** Cytoplasmic male sterility; fertility restorer genes; SCAR; orf; Brassica.

## Introduction

Heterosis breeding is a most promising strategy for enhancing crop productivity in *Brassica juncea*, a major oilseed crop of the Indian subcontinent. Relevance of divergent gene pools for heterosis breeding has already been shown in *B. juncea* (Pradhan *et al.,* 1993; Srivastava *et al.,* 2000). However, besides the identification of suitable combiners, a pollination control mechanism for the production of hybrid seed on a large scale is required for the success of the technology. Cytoplasmic Male Sterility (CMS), a phenotypic manifestation of incompatibility between nuclear and cytoplasmic genomes, is a maternally inherited trait that has been successfully exploited as an effective pollination control mechanism for the production of hybrid seed in many crops (Liu *et al.,* 2001; Havey 2004). CMS is a widespread and classic non-Mendelian trait that results from rearrangements of the mitochondrial genome (Bonen and Brown, 1993). As the CMS plants are incapable of self-pollination, when these are planted alongside a male-fertile line, all the seed that forms on the sterile plants are essentially the hybrid of the two parents. Due to maternal transmission of CMS, however, one might expect the hybrids to be male sterile, which would be problematic for seed crops such as canola. Fortunately, specific dominant nuclear genes termed restorers of fertility (R*f*) have been identified that can suppress the male-sterile phenotype and restore fertility to $F_1$ hybrids. The components of a CGMS system, therefore, consist of the CMS line, that contains the male sterile cytoplasm (or mtDNA) and lacks functional nuclear fertility restorer gene/allele. The maintainer line, that contains a fertile or normal mtDNA but is isogenic with the CMS line at nuclear genetic loci, and a restorer line, that usually contains the male sterile mtDNA but is homozygous for the dominant nuclear R*f* gene (Bhat *et al.,* 2005). In a number of cases, CMS is often associated with novel mitochondrial open reading frame (*orf*) generated by rearrangement and recombination of mitochondrial genes (Hanson and Bentolila, 2004; Schenable and Wise, 1998).

The application of this trait in the production of hybrid seeds in crops has been widely explored since its discovery in the beginning of the Twentieth century. CMS has been successfully used for production of hybrid seeds in the *Brassica* species. Therefore, many researchers use *Brassica* crops to analyze the molecular mechanism underlying CMS in plants. A stable CMS line of *B. juncea* was derived from the somatic hybrid *Moricandia arvensis* + *B. juncea* followed by back crosses with *B. juncea* (Prakash *et al.,* 1998). The fertility restorer gene for this CMS was introgressed into *B.juncea* from *M. arvensis*. Subsequently, it was found that this *Rf* locus is capable of restoring male fertility to two other CMS systems of *B. juncea* carrying *Diplotaxis catholica* or *Diplotaxis erucoides* cytoplasm (Bhat *et al.,* 2005, 2006).

At present, two different approaches are adopted for development of hybrids. The

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first approach is called three-line system involving a CMS line, a maintainer line and restorer line. This is the most popular method worldwide in almost all the crops in which hybrids have been developed and commercialized. CMS is a maternally inherited trait that results in the inability of the plant to produce fertile pollen. Pollen fertility is restored by nuclear-encoded genes called fertility restorer (R*f*) gene. The second approach is called two-line system involving environmentally sensitive male sterility. Hybrids of this kind are grown to a limited extent only in China. DNA-based molecular markers tightly linked to fertility restorer genes have also been identified in several other species such as rice (Zhang *et al.,* 1997), petunia (Bentolila *et al.*, 2002), sorghum (Wen *et al.,* 2002), barley (Matsui *et al.,* 2001), sunflower (Horn *et al.,* 2003), cotton (*Gossypium hirsutum*; Liu *et al.,* 2003), wheat (Zhou *et al.,* 2005) and chili pepper (Kim *et al.,* 2006). The present study was undertaken to validate the markers linked to sterility and fertility restorer gene fertility restorer gene of CMS (*B. juncea*) markers linked to the *Rf* locus, which is useful for identifying the restorer in segregating populations and in seed purity evaluation and management.

## Materials and Methods

The CMS line-MJA5 (A line, *Moricandia* based), maintainer line-MJB5 (B line), restorer line-MJR-1 (R line) and hybrid (AxR) seed used in the present study were obtained from the Directorate of Rapeseed and Mustard Research (DRMR), Bharatpur, India. Young leaves were used for genomic DNA isolation from A, B, R and AxR lines, from the plants grown in the National Phytotron Facility, Indian Agricultural Research Institute, New Delhi.

**DNA extraction:**

DNA extraction was performed by the microextraction method described by Prabhu *et al.,* (1998). DNA was quantified spectro-photometrically, and diluted accordingly to a final concentration of 10ng/µl for PCR analysis.

**PCR amplification using SCAR and orf103 primers:**

The PCR was carried out in a final volume of 25µl containing 1µl DNA template (20-50ng/µl), 0.5µl dNTPs (10mM, 2.5mM each), 2.5µl 10x PCR buffer containing $MgCl_2$ (25mM), 0.3µl *Taq* polymerase (1U/µl), 1µl of each of the two primers (forward and reverse, 20ng), 18.7µl nuclease-free water. *orf108* and SCAR-3 primers reported to be linked to R*f* genes were used for validating linkages of these markers for sterility and fertility in A, B, R and AxR lines. The thermocycler (Eppendorf Company) was programmed as follows: 4 min at 94°C; 36 cycles of 45s at 94°C, 1 min at 55°C, and 1 min at 72°C and a final 7 min extension at 72°C, followed by cooling down to 4°C. The markers were checked twice for their reproducibility. The polymerase chain reaction products (25µl) were mixed with 1X gel loading buffer (3µl) and loaded onto an metaphor (3%, w/v) gel in 1X TAE (Tris-aceticacid-EDTA) buffer, and electrophoresis was performed at 70 V for 180 min. The gel was stained in an ethidium bromide solution (2µl/100 mL 1X TAE buffer) for 40 min, and the bands were visualized under UV in a Gel Doc Image Analysis System (Alpha Innotech Corporation). The sizes of the amplified fragments were estimated with the help of 100bp DNA size standards (MBI Fermentas).

## Results and Discussion

When SCAR-3 primers were used in PCR, the A and B lines produced no bands, while the restorer line and the $F_1$ (AxR) produced bands of 200bp with the SCAR primer (Fig.1). The primer SCAR-3 used in this study was reported to be linked to Rf gene for s-type cytoplasm. In CMS (*Mori*) *B. juncea*, male sterility is associated with altered expression of the mitochondrial *atp α* gene. In floral buds of male sterile plants, a 2,000nt long *atp α* transcript is detected which is reduced to 1,800nt in fertility restored plants (Gaikwad *et al.,* 2006). The level of expression of *orf 108* was detected at 900 bp amplicon, obtained in sterile A line, R line and AxR (Fig.1&2), whereas no amplification occurred in B line (fertile). This would be seen as the male fertile $F_1$ hybrid which has heterozygous restorer gene and CMS-associated transcripts in male gametes, which would ultimately abort a lower intensity band of *orf 108* was expected in the $F_1$ hybrid plants whereas, it was completely absent in homozygous plants.

Identification of linked marker (s) are useful in map-based cloning of fertility restorer genes and to understand the possible role of *Rf* genes in other biological processes. SCAR-3 primer amplified at 200bp amplicon in R and AxR lines was similar to that

reported by Ashutosh *et al.,* (2007) it was also reported 200bp SCAR-3 amplicon shared homology with *B. rapa* genome sequence. However, in the present study, SCAR-3 primer amplified an additional 400 bp fragment in all fertile plants suggesting that the two SCAR-3 fragments are tightly linked. The marker fragments were found to be linked to the fertility restorer gene and mapped on one side of the *R*f locus. The SCAR-3 marker was found to be tightly linked to the *R*f locus with a map distance of 0.6 cM. The previous report of Jing et al. (2001) reported three rice nuclear fertility restorer genes (Rf-1, Rf-4 and Rf-5(t)) for three types of CMS (BT, WA and HL) which could be allelic and different alleles or haplotypes of a single nuclear locus that can restore the fertility of different types of CMS. This also implies that the markers developed for Rf-1 could be applicable for Rf-4 and vice versa.

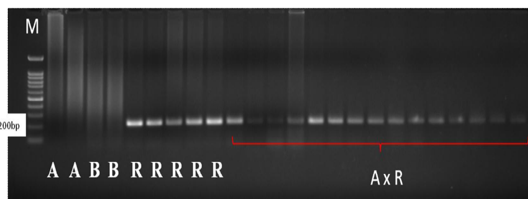


**Fig.1:** Confirmation of presence of Rf pattern association with R (restorer) and $F_1$ (AxR) lines by SCAR-3 primer. A: sterile line, B: maintainer line, M: marker 100 bp

**Fig.2:** Validation of PCR amplification of CMS (A) (s-cytoplsam), maintainer (B) lines and restorer (R) lines using *orf108*. M: marker 100 bp

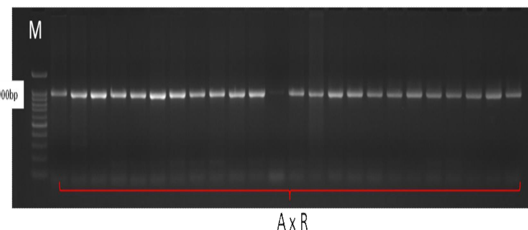


**Fig.3:** Screening results of PCR amplification of $F_1$ indiviuals (AxR) lines using *orf108*. M: marker 100 bp

Akagi *et al.* (1996) reported that the marker pRf1 and 2 amplify 345 and 329 bp DNA fragments in R and A lines, respectively. Expression of *orf 108* was detected at 900 bp amplicon in sterile A line, R line and AxR, whereas no amplification occurred in B line (fertile). Gaikwad et al. *(*2006) reported that *atpA* transcripts in flower bud tissues of CMS plants are longer than that in normal *B. juncea*, *M. arvensis* and the fertility-restored plants. The genetic studies revealed that the Rf gene acts gametophytically to confer male fertility to the CMS plants (Ashutosh *et al.,* 2007). Upstream region of *atpA* (1,728–2,173 of the 6.0 kb clone) had revealed a single transcript of 2,800 nt only in the CMS line, indicating that the *atpA* transcript of the CMS line differs from normal *B. juncea* in the 50 region. Results of Xiaolin et al. (2010) suggest that the *atpA* gene and *orf324* gene fragments located in the DNA region may be associated with the CMS in plants of the genera *Brassica* and *Raphanus*. An additional, 663-bp fragment of the *orf220* gene was identified only in the *B. juncea* CMS lines. This result indicates that the *B. juncea* CMS line is unique and the 663-bp DNA fragment of the *orf220* gene region might play a key role in the characterization of CMS in tuber mustard.

Hence, the present study validated the application of this marker for identifying the restorers in segregating populations, which can accelerate breeding of restorer lines and can hence enhance efficiency in hybrid breeding in *B. juncea*. It can also be applied for seed purity evaluation and management by eliminating the contamination in hybrid seed stocks at the seedling stage itself (Yashitola *et al.,* 2002). This marker can be used to test the purity of hybrids as an effective substitute to the time consuming and laborious grow-out test (GOT).

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