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Dr. Geetika Malik
Scientist (Vegetable Science) and Head, Division of Vegetable Science, ICAR-Central Institute of Temperate Horticulture, Srinagar, Jammu and Kashmir, India

Dr. Ajmer Singh Dhatt
Professor (Vegetable Science) and Additional Director of Research, PAU, Directorate of Research, Punjab Agricultural University, Ludhiana, Punjab, India

Dr. Vishal Dinkar
Scientist, Department of Genetics and Plant Breeding, ICAR-Central Institute of Temperate Horticulture, Srinagar, Jammu and Kashmir, India

Corresponding Author:
Dr. Geetika Malik
Scientist (Vegetable Science) and Head, Division of Vegetable Science, ICAR-Central Institute of Temperate Horticulture, Srinagar, Jammu and Kashmir, India

Validation of linkage disequilibrium between *PsaO* marker and nuclear male fertility restorer locus in north Indian onions (*Allium cepa* L.)

Dr. Geetika Malik, Dr. Ajmer Singh Dhatt and Dr. Vishal Dinkar

Abstract
The DNA markers play crucial role in economic and rapid development of CGMS lines in onion. One such marker named *PsaO* is reported to be linked with nuclear male fertility restorer locus (*Ms/ms*) in onion. This marker was used to screen three North Indian onion cultivars *viz.,* Punjab Naroya, Punjab Selection and Punjab White already genotyped for cytoplasmic type. The *PsaO* marker effectively identified various alleles of the locus in all the populations, thereby, ascertaining its ability to reveal polymorphism. However, on morphological scoring, considerable deviations in its allelic frequencies from the frequencies divulged by *PsaO* were observed. The difference in the observed frequencies for *msms* allele was 0.25, 0.11 and 0.18 in Punjab Naroya, Punjab Selection and Punjab White, respectively. For *Msms*, it was 0.30 and 0.06 for Punjab Naroya and Punjab White, respectively, however no deviation for Punjab Selection was observed. For *MsMs* allele the deviations were 0.05, 0.11 and 0.12 for Punjab Naroya, Punjab Selection and Punjab White, respectively. This reveals a lack of strong linkage between the marker and the gene it has been developed for, with respect to North Indian onion populations. Therefore, the study advocates development of more tightly linked, gene-based, or a functional marker for male fertility restorer locus in North Indian onion.

Keywords: Cytoplasmic genic male sterility, Linkage, *Ms/ms* locus, onion hybrid, *PsaO* marker

1. Introduction
Onion (*Allium cepa* L.) is among world's most cultivated vegetables. Onion is historically popular for therapeutic properties like anti-yeast (Dankert *et al.*, 1979) [4], anti-allergic bronchial asthma (Dorsch *et al.*, 1988) [5], anti-platelet, lipid lowering, anti-thrombotic (Bordia *et al.*, 1996) [3], anti-fungal (Yin and Tsao, 1999) [14], anti-hyperglycemic and anti-cancerous (Gao *et al.*, 1999) [7]. India is its second largest producer and among the foremost exporters. To cater to the needs of importing countries, F_1 hybrid breeding has become growing trend in onion breeding programs in India, as it is an effective technique to customize onions for color, shape and size besides obtaining higher productivity and product uniformity. However, in the absence of male sterility (MS), it is impractical to produce onion F_1 hybrids since innumerable small florets limit manual emasculation. MS in onion follows cytoplasmic genic male sterility (CGMS) system in which cytoplasm can be S or T instead of male fertile N cytoplasm (Berninger, 1965) [2]. In contrast to S-cytoplasmic plants, which are exclusively MS when the nuclear *Ms* locus is homozygous recessive, plants with N cytoplasm are MF regardless of the nuclear *Ms* locus (Jones and Clarke, 1943) [9]. Due to monogenic inheritance and stability over environmental conditions, S-cytoplasm has been used more extensively but three loci inheritance of T-type male sterility is complicated (Jones and Clark, 1943; Schweisguth, 1973; Havey, 2000) [9, 12, 8].

Thus, it is evident that identification of cytoplasms and determination of *Ms/ms* locus are to be done to develop CGMS lines (MS/S*msms* and maintainer lines/N*msms*) in populations having S cytoplasm. However, due to onion's biennial lifespan, it takes 4–8 years to accomplish this morphologically through crosses and progeny testing. DNA based molecular markers offer an attractive opportunity to the onion breeders since they are capable of determining cytoplasm and fertility restorer locus at the seedling stage itself. Conclusively, DNA based markers are presently the most economical and fastest tool to develop CGMS lines in onion. With a view to utilize molecular markers in developing CGMS lines, we tested marker *PsaO* developed by Bang *et al.* (2011) [1] on North Indian onion cultivars already identified for the presence of S-cytoplasm (Sheemar and Dhatt, 2015; Malik *et al.*, 2017) [13, 10].

The testing involved amplification of individual plant DNAs from each population with the mentioned marker for revealing the *Ms/ms* locus condition followed by validation of results with conventional method of identification that involves crossing with a male sterile (S*msms*) individual and subsequent genetic scoring of progeny.

2. Materials and Methods

2.1 Experimentation Site and Plant material

The study was conducted at Experimental Farm and Plant Pathology Laboratory of Division of Vegetable Science, Punjab Agricultural University (PAU), Ludhiana, India. The varieties Punjab Naroya, Punjab Selection and Punjab White and MS line 97-A were developed and being maintained at the Division of Vegetable Science, PAU.

A known male sterile line 97A and three open pollinated cultivars *viz.,* Punjab Naroya, Punjab Selection, and Punjab White were used for molecular genotyping with *PsaO* marker, as well as to perform test crossings for validation of the result of screening. Three test cross sets were made each having 97A paired with one population. In each set, 200 bulbs of the population were planted for pair crossing with 200 bulbs of 97A. All one-on-one pairs were distanced from each other by 60 cm within the row. Paired rows were spaced at 120 cm for the ease of pollination. To avoid contamination from foreign pollen, all test cross sets were protected by 40-mesh agro-net walking tunnels just before flowering.

2.2 Molecular Analysis

From each population, genomic DNA of 125 individuals was isolated by CTAB method describes by Doyle and Doyle (1987) [6] with some modifications. The quantity and quality of genomic DNA samples were checked with spectrophotometer (Nanodrop 1000, Thermo Scientific Inc., USA). The genomic DNA was stored at –20 °C for future use. To determine cytoplasm type (S or N) of selected plants, DNA amplification with molecular markers was done as suggested by Sato (1998) [11]. The PCR was performed in a reaction mixture comprised of 50 ng DNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.25 µM of each primer and 3 units/µl Taq Polymerase. The PCR involved initial denaturation at 94 °C (2 minutes), followed by 35 cycles of denaturation at 94 °C (30 seconds), primer annealing at 53 °C (2 minutes), extension at 72 °C (2 minutes) and final extension at 72 °C for 10 minutes.

To genotype nuclear restorer locus of these plants, *PsaO* marker was used (Bang *et al*., 2011) [1]. Each 10 µl PCR reaction mixture, 50 ng genomic DNA, 0.6 µl of 25 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each of reverse and forward *PsaO* primers and 3 units/µl Taq Polymerase were used. After initial denaturation, the temperature profile included first 10 cycles from denaturation to annealing to extension at 95 °C (30 seconds), 95 °C (30 seconds) and 72 °C (2 minutes), respectively, with 0.8 °C decrement in each cycle. In the second part, denaturation at 95 °C (30 seconds) was followed by annealing at 57 °C (30 seconds) and extension at 72 °C (2 minutes) followed by a final extension at 72 °C (7 minutes). The PCR products were separated by horizontal electrophoresis in 3% Agarose and scored against 100 bp ladder DNA. Gels were visualized using UV-transilluminator gel documentation system.

2.3 Phenotyping

For morphological confirmation, the plants that were genotyped by linked markers for cytotype and fertility restorer locus were also test crossed with MS line 97A. For cross pollination of MS plant and during selfing of corresponding MF plant of each pair soft paint brushes were used. Proper precautionary measures such as hand washing after each pollinating each pair and sterilized brushes were taken to avoid contamination of pollen from neighbour plants. Crossed seed of MS and selfed seed of MF plants were harvested separately. From the progeny of each test cross (i.e., seeds resulting from cross and borne on MS plant) 25 plants were transplanted. To know fertility restoration status of parent cultivars, each progeny was scored upon dehiscence of the pollen. The plants whose 100 per cent progeny was MS was designated as maintainer (N*msms*), one whose 50 per cent progeny was male sterile was designated as heterozygote (N/S*Msms*) and the one with all MF progeny was classified as dominant homozygote (N/S*MsMs*). The results obtained were compared with those of *PsaO* marker.

3. Results and Discussion

In onion, *OPT* and *PsaO* markers were mapped at 1.5 cM and 6.2 cM distance to the fertility restorer locus (*Ms/ms*), respectively (Bang *et al.,* 2011) [1]. In conjunction with cytotype markers, they can very effectively be utilized for developing CGMS lines in onion. In the present study, individual plants of three North Indian onion populations *viz.,* Punjab Naroya, Punjab Selection and Punjab White with already known for cytotype (N or S) were screened with *OPT* and *PsaO* markers on individual plant basis. The *OPT* marker, however, did not show amplification for *Ms/ms* locus in any of the three populations. Therefore, only *PsaO* marker was further used. Marker amplification pattern of 100 plants in each population with mitochondrial DNA based *cob* marker revealed the presence of S cytoplasm in addition to N cytoplasm. Amplification of nuclear DNA marker *PsaO* helped in determining the fertility restorer locus. The combined documentation of cytoplasmic and nuclear marker amplifications revealed the number of MS (S*msms*), maintainer (N*msms*), heterozygotes (N/S*Msms*) and fertility restorer (N/S*MsMs*) plants in each population (Fig. 1).

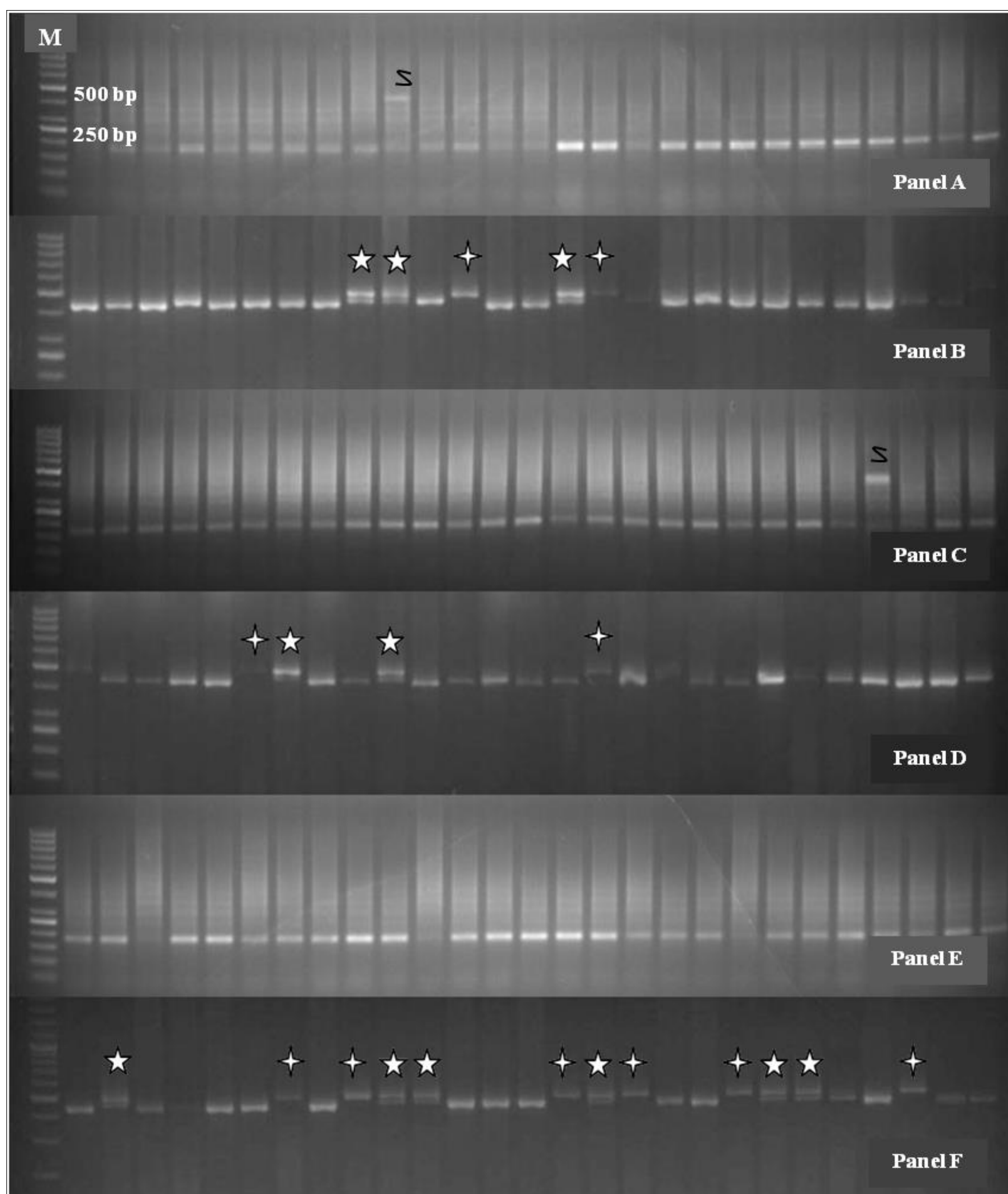

Fig 1: PCR profiles of Punjab Naroya (Panel A), Punjab Selection (Panel C) and Punjab White (Panel E) with *cob* marker for cytotype identification and of Punjab Naroya (Panel B), Punjab Selection (Panel D) and Punjab White (Panel F) with *PsaO* marker for *Msms* locus determination. M: 50 bp DNA ladder

Panel A, C and E			Panel B, D and F		
S	:	S cytoplasm = 414 bp	✧	:	*MsMs* = 490 bp
Others	:	N cytoplasm = 180 bp	☆	:	*Msms* = 437 bp + 490 bp
			Others	:	*msms* = 437 bp

Marker based analysis in Punjab Naroya, Punjab Selection and Punjab White derived populations showed the presence of both PsaO490 and PsaO437 haplotypes, either as a single band or together as reported polymorphism for the locus. However, PsaO454 haplotype was not detected in our study. The marker-based analysis of Punjab Naroya population revealed 85 plants were maintainers (N*msms*), 10 heterozygous MF (N*Msms*) and 5 fertility restorers (N*MsMs*). Likewise, in Punjab Selection population, 1 plant was MS (S*msms*), 74 plants were maintainers, 14 plants were heterozygous MF and 11 plants were fertility restorers. And in Punjab White population analysis revealed 1 plant was MS, 78 plants were maintainer, 9 plants were heterozygous MF and 12 plants were fertility restorers.

Test cross analysis were conducted for molecular marker based identified maintainers (N*msms*), heterozygotes (N/S*Msms*) and fertility restorers (N/S *MsMs*) individually with a known male sterile line 97A. This revealed the true genetic status of these plants irrespective of that revealed by the marker and divulged the actual frequencies of different alleles in the populations. The scoring of test cross progenies for the nuclear status of *Ms/ms* locus of each plant revealed that the Punjab Naroya possesses *msms* and *Msms* alleles at frequencies of 0.6 and 0.4, respectively. In Punjab Selection,

msms and *Msms* alleles exist at frequencies of 0.86 and 0.14, respectively; and in Punjab White have *msms* and *Msms* frequencies of 0.97 and 0.03, respectively. Further, there is complete absence of *MsMs* allele in all of these populations. Table 1 shows the allelic frequencies of *Ms/ms* locus as revealed by *PsaO* marker as well as test crossing and the differences thereof can be compared easily. Despite the differences, both the methods of allele identification have revealed the most frequent presence of *msms*, followed by *Msms* and lowest frequency of *MsMs* allele in the populations. Thus, despite that *PsaO* marker cannot predict the exact *Ms/ms* allelic frequencies in three populations studied, it can figure out the proportion of non-restorer, heterozygous and restorer plants to a considerable accuracy.

Table 1: Frequencies of *msms, Msms* and *MsMs* genotypes revealed by *PsaO* marker and test-crossing plus progeny scoring.

Population	Allelic frequencies					
	By *PsaO* marker			By test crossing and progeny scoring		
	msms	*Msms*	*MsMs*	*msms*	*Msms*	*MsMs*
Punjab Naroya	0.850	0.100	0.050	0.600	0.400	0.000
Punjab Selection	0.750	0.140	0.110	0.860	0.140	0.000
Punjab White	0.790	0.090	0.120	0.970	0.030	0.000

Also, unlike many other accessions of onion from other parts of the world, North Indian populations did not show the presence of PsaO454 haplotype. The reason for discrepancy in results can be a recombination event between *PsaO* marker and *Ms/ms* in the studied populations, which may have arisen due to linkage disequilibrium decay over generations or these populations probably never, had either physical or genetic linkage between *PsaO* marker and *Ms/ms* locus in the first place. This observation hints at the genetic divergence of North Indian populations from many of the rest of the world, which may have occurred due to geographical and reproductive isolation.

4. Conclusion

Our findings pin point that much closely linked and robust marker(s) need to be developed for precise prediction by genotyping of the *Ms/ms* locus in North Indian onions. Alternatively, *OPT* marker can be used for accurate molecular analysis of plants by establishing a novel amplification pattern in the parents (Bang *et al.* 2011) [1]. Thus, a more reliable marker system can pace up the onion molecular breeding programs and hybrid development.

5. Author's contribution

Conceptualization of research (ASD and GM); Designing of the experiments (ASD and GM); Contribution of experimental materials (ASD); Execution of field/lab experiments and data collection (GM); Analysis of data and interpretation (GM and ASD); Preparation of the manuscript (GM and VD).

6. Declaration

The authors declare no conflict of interest.

7. Acknowledgement

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