Molecular Biology Reports (2022) 49:5535–5545
https://doi.org/10.1007/s11033-022-07451-9

ORIGINAL ARTICLE

Molecular marker-based characterization of cytoplasm and restorer of male sterility (*Ms*) locus in commercially grown onions in India

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Received: 20 February 2022 / Accepted: 5 April 2022 / Published online: 11 April 2022

Abstract

Background The cytoplasmic-genic male-sterility system has been extensively employed for the production of onion hybrids. Molecular marker-assisted characterization of the cytotypes and genotyping at the restorer-of male-fertility (*Ms)* locus is important for the accelerated breeding of onion hybrids. Indian onion breeding has focussed more on open-pollinated varieties than hybrids. To accelerate the breeding efforts, marker-assisted selection (MAS) plays a pivotal role.

Methods and results This study aimed to characterize the Indian breeding lines, varieties, hybrids, and exotic accessions for cytotype and *Ms* locus. For cytoplasm, cytotype markers, *acc*D, and MKFR and for *Ms* locus identification, PCR markers *AcPMS1* and *AcSKP1* were employed. Bulk strategy to identify cytoplasm and *Ms* locus was tested. Sequencing of PCR products amplified by *acc*D was also tried. Both the *acc*D and MKFR were synonymous in cytoplasm identification except in T821 where T cytoplasm was identified. *AcPMS1* was more reliable than *AcSKP1* for *Ms* locus identification. Sequencing proved that N and T cytoplasm are identical. Bulking strategy can be used for cytotype identification but not for *Ms* locus.

Conclusions Indian onions have a predominance of normal (N) cytoplasm and homozygous recessive (*msms*) locus. This might be beneficial for hybrid development. S cytoplasm was identified in exotic varieties. For the first time, T cytoplasm has been reported from India. These findings will assist Indian onion breeders to develop MAS strategies for accelerating hybrid development programs. And for the release of onion hybrids with high productivity and uniformity.

Keywords Onion. *Allium cepa* · Cytoplasmic male-sterility · Restoration of male fertility · Heterosis

Introduction

Onion (*Allium cepa* L.) is an extremely important and indispensable element in almost every culture as a vegetable crop. It is consumed year long and is not limited to a particular season, unlike other vegetables. Globally, amongst the top ten vegetable crops produced, onion is at second place and ranks third in the exported fresh vegetable crop category [1]. Onion dominates the local India export trade (> 50%) share with a foreign exchange value of US$ 336 million [2]. India leads in the area under cultivation and production with an area of 1.43 million hectares and a production of 26.74 million metric tonnes. But the productivity is less (18.6 t/ha) as compared to other countries like the Republic of Korea (79.6 t/ha), the USA, Australia, Spain, etc. [3]. Low productivity may be ascribed to the lack of hybrids (< 1%) in the Indian market compared to the 98% market share of hybrids in the USA, UK, Italy, Netherlands, etc. Studies in onion have some challenges owing to some of its features like its biennial nature, high inbreeding depression, the requirement of isolation distances to maintain genetic uniformity, and environmental factors.

Heterosis of hybrids for desirable utilitarian traits like yield, bulb weight, height, the diameter has been acknowledged and commercially exploited [4, 5]. In India, the public sector released two hybrids (Arka Kirtiman and Arka Lalima) but they were not high-yielding on the national level [6]. Hybrid onions are cherished by farmers and consumers throughout the world for their potential higher yield

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and uniformity in a bulb shape, size, color, and maturity. Research on onion hybrids was initiated with the discovery of 'S' cytoplasm [7] in cultivar 'Italian Red' and restorer of fertility (*Ms*) locus [8]. S cytoplasmic male sterility is favored worldwide due to its stable nature under multi-locations and maintenance by a single '*Ms'* locus [9]. Since then, the phenomenon of male sterility has been reported in more than 140 species. Berninger [10] reported another type of male sterility, CMS-T, wherein the fertility restoration was controlled by three independent loci [11]. Male sterility in the CMS-S system is maintained by crossing a sterile plant with S cytoplasm and homozygous recessive (*msms*) loci to a plant with N cytoplasm and *msms* locus. Whereas the restoration of the fertility can be obtained by crossing the S cytoplasmic plants with a plant having either '*Msms'* or '*MsMs'* genotype. Conventionally, the genetic constitution of the parent plants can be identified by crossing a male sterile plant with a fertile plant via test crossing and then studying the F1 to determine the parentage also known as progeny testing [12].

Highly cross-pollinated nature coupled with high inbreeding depression and biennial generation time lengthens the period required for the development of onion inbreds with the known genetic constitution. Employment of PCR markers for the determination of cytoplasm and nuclear *Ms* locus is highly recommended for accelerated hybrid development. PCR markers identifying normal (N) and sterile (S) cytoplasm [13–15] and T cytoplasm [16–18] have been reported. Furthermore, Kim et al. [19] described the presence of cytotype X and Y based on the comparative analysis of chloroplast genome of male fertile and male-sterile cytoplasm. Marker-assisted selection of nuclear *Ms* locus was made possible with the development of RFLP marker [20], CAPS markers [21, 22], SCAR marker [23], SNPs [24] followed by other PCR markers [19, 25–27]. Till 2015, cytoplasm markers reported by Sato [14] and Engelke et al. [16] were mostly sought-after markers [28–30]. Recently, new primers for cytoplasm [15, 18] and *Ms* locus [19, 27] are being utilized for the identification of cytoplasm and *Ms* locus [6, 28, 31] (Table 1). Although reported to be in complete linkage disequilibrium with *Ms* locus, still new PCR markers linked to *Ms* locus are being reported. Khar and Saini [6] reported about the limitation of these PCR markers in open-pollinated populations and Khrustaleva et al. [32] hypothesized that limitations may be due to PCR markers assigned to the centromere of Chromosome 2 which is a region of lower recombination. New studies suggest a novel cytotype Y containing a unique stoichiometry of *cox1* and *orf725* [33], 'T like' designated as 'R' [34], and a novel haplotype of *Ms* locus [35] and a new Ms2 locus situated at 70 cM away from the *Ms* locus on the end of chromosome 2 [36]. To sum up, presently there are three male-sterile cytoplasms reported in onion. The first cytoplasm is the S cytoplasm with one [8] or possibly two [36] nuclear restorer loci. The second one is the T cytoplasm with a complex male-fertility restoration that is maintained possibly by three complementary loci [11]. And the third latest one is the R cytoplasm which appears to be restored by the *Ms* locus [34].

Our study aimed to characterize the commercially grown open-pollinated varieties (OPVs), breeding material, and

Table 1 List of the major PCR markers used for determination of cytoplasm and *Ms* locus in onion

Name	Type	Primer	Orientation (5'-3')	Identifier	Band Size	References
cob	PCR	*cob*-N	TCTAGATGTCGCATCAGTGGAATCC	Cytoplasm	N-180 bp, S-414 bp	[14]
		cob-S	GTCCAGTTCCTATAGAACCTATCACT			
		cob-C	CTTTTCTATGGTGACAACTCCTCTT			
MKFR	PCR	MKF	CATAGGCGGGCTCACAGGAATA	Cytoplasm	N-833 bp, S-628 bp	[18]
		MKR1	AATCCTAGTGTCCGGGGTTTCT			
		MKR2	CAGCGAACTTTCATTCTTTCGC			
*acc*D	*Indel*	*acc*DF	AGAATGAGGAGCAGGAAAACTCT	Cytoplasm	N-375 bp, S-420 bp	[15]
		*acc*DR	AGTCGTGATTGTTACTCTTAGACCT			
OPT	PCR	OPTF	CCTTGGAAAGGCGCAACTAAAGATTTGA	*Ms* locus	*MsMs*-659 bp, *msms*- 526 bp	[25]
		OPTR	TGTGGCCCAATAATACAAACAAGCAGGA			
Jnurf13	*Indel*	jnurf13F	TGCAAGCTTGGAACTTACGC	*Ms* locus	*MsMs*-241 bp, *msms*-249 bp	[26]
		jnurf13R	TTGCCAAAGGTTGCAATACA			
AcSKP1	Multiplex	FU898	GCAATACACAGCTTCTAGCTGAATT	*Ms* locus	*MsMs*-898 bp, *msms*-628 bp	[27]
		FD898	AACACACACACAGAGTGAGAAATTTTATATAT			
		SU628	TCTGTGTGTGTGTGTAATTTCTCTG			
		SD628	CGGAAGATTAATATTTTGCGTATACAT			
AcPMS1	PCR	*AcPMS1*F	GGTCACCAGGTGGATAGAGAA	*Ms* locus	*MsMs*-242 bp, *msms*-276 bp	[19]
		*AcPMS1*R	TCATTGAGCTGCATCCAAAA			

hybrids marketed in India. Also, our purpose was to assign the genetic constitution to the breeding lines for the future hybrid breeding programs and to ascertain the genetic architecture of OPVs and hybrids available in the market so that a blueprint for heterosis breeding program can be outlined. For identification of cytoplasm, two PCR markers specific for onion cytoplasm viz., *acc*D, an indel marker [15] and MKFR designed on a chimeric gene *orf725* [18] were utilized. For the *Ms* locus determination, two PCR markers reported for the restorer-of-male-fertility (*Ms*) locus namely AcSKP1 [27] and *AcPMS1* [19] were employed.

Materials and methods

Plant materials

Eighteen onion accessions including two commercially grown open-pollinated varieties (OPVs) (Pusa Red, Pusa Madhavi), four breeding lines (POS13, POS14, POS21, POS22), nine doubled haploid (DH) lines (PMDH2, PMDH3, PMDH7, Ha2hi3.1.2, Hap2hi3.1.4, Hap2hi3.1.5, Hap2hi3.1.6, Hap2hi3.1.7 and Hap2hi3.1.8), and three hybrids (Juni, Matahari, T821) were used for determining the onion cytotype and the genotype at *Ms* locus. Hybrids, popular among the farmers for high productivity, were purchased from the market. Additionally, twenty-five accessions of commercially grown OPVs including four 4 exotic accessions were also investigated for this study. All the onion bulb material was planted during the *rabi* (winter) season under net house conditions in the experimental field of the Division of Vegetable Science, Indian Agriculture Research Institute, New Delhi, India.

DNA extraction and PCR amplification

The genomic DNA from 626 individual plants of 18 onion accessions (Table 2) and 208 individuals from the OPVs and exotic lines (Table 3) was extracted individually from young leaves using the CTAB method [37]. DNA integrity was examined by agarose gel electrophoresis and quantified by a Nanodrop spectrophotometer. The DNA quantity of the samples was adjusted to a 10 ng/µl concentration and then stored at − 20 °C until PCR analysis. DNA amplification was performed in a programmable thermal controller (Eppendorf Vapo Protect Mastercycler Pro) in a 10 µl volume of reaction mixture containing 5.0 µl Dream Taq™ Green PCR (Thermo Fisher Scientific Inc., USA), 1.0 µl genomic DNA and 1.0 µl

Table 2 Cytoplasm and *Ms* locus distribution in breeding lines (BL), open-pollinated varieties (OPV), doubled haploids (DHs), and hybrids in onion

S. No	Accession	Nature	Plants	Cytoplasmic marker					*Ms* locus markers					
				*acc*D		MKFR			AcSKP1			*AcPMS1*		
				S	N	S	N	T	*MsMs*	*Msms*	*msms*	*MsMs*	*Msms*	*msms*
1	POS13	BL	14	14	0	14	0	0	0	0	14	0	0	14
2	POS14	BL	22	0	22	0	22	0	0	0	22	0	0	22
3	POS21	BL	25	25	0	25	0	0	0	0	25	0	0	25
4	POS22	BL	25	0	25	0	25	0	0	0	25	0	0	25
5	Pusa Red	OPV	67	24	43	24	43	0	0	0	67	0	16	51
6	Pusa Madhavi	OPV	20	2	18	2	18	0	0	0	20	0	0	20
7	PMDH2	DH	36	0	36	0	36	0	0	0	36	0	0	36
8	PMDH3	DH	9	0	9	0	9	0	0	0	9	0	0	9
9	PMDH7	DH	16	0	16	0	16	0	0	0	16	0	0	16
10	Hap2hi3.1.2	DH	42	0	42	0	42	0	0	0	42	0	0	42
11	Hap2hi3.1.4	DH	11	0	11	0	11	0	0	0	11	0	0	11
12	Hap2hi3.1.5	DH	47	0	47	0	47	0	0	0	47	0	0	47
13	Hap2hi3.1.6	DH	69	0	69	0	69	0	0	69	0	0	0	69
14	Hap2hi3.1.7	DH	54	0	54	0	54	0	0	54	0	0	0	54
15	Hap2hi3.1.8	DH	23	0	23	0	23	0	0	0	23	0	0	23
16	Juni	Hybrid[1]	74	74	0	74	0	0	0	0	74	0	8	66
17	Matahari	Hybrid[1]	25	20	5	20	5	0	0	0	25	0	18	7
18	T821	Hybrid[2]	47	0	47	9	0	38	0	0	47	0	47	0
		Total	626	159	467	168	420	38	0	123	503	0	89	537

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[2]Takii Seeds, Japan

Table 3 Cytoplasm and *Ms* locus distribution in commercial Indian open-pollinated varieties (OPV), selections, and exotic onions

S. No	Genotype	Status	Plants	Cytoplasm (*acc*D)		*Ms* Locus (*AcPMS1*)		
				N-	S-	*MsMs*	*Msms*	*msms*
1	ALR	OPV	8	8	0	0	0	8
2	Arka Bindu	OPV	8	8	0	0	0	8
3	Arka Kalyan	OPV	8	3	5	0	1	7
4	Arka Kirtiman	OPV	8	8	0	0	0	8
5	Bhima Red	OPV	8	8	0	0	0	8
6	Bhima Shubra	OPV	8	8	0	0	0	8
7	Hisar3	OPV	8	8	0	0	0	8
8	JNDWO85	OPV	8	8	0	0	0	8
9	JWO207	OPV	8	8	0	0	0	8
10	JWO608	OPV	8	8	0	0	0	8
11	JWO808	OPV	8	8	0	0	0	8
12	L355	OPV	8	8	0	0	0	8
13	N241	OPV	8	8	0	0	0	8
14	Phule Safed	OPV	8	8	0	0	0	8
15	Phule Samarth	OPV	8	8	0	0	0	8
16	Phursungi Local	OPV	8	8	0	0	0	8
17	Pillipati Junagarh	OPV	8	8	0	0	0	8
18	VL Pyaz	OPV	16	10	6	4	10	2
19	Sel.397	Selection	8	8	0	0	0	8
20	Sel157	Selection	8	2	6	1	3	4
21	Sel402	Selection	8	8	0	0	0	8
22	NBPGR2	Exotic	8	8	0	0	2	6
23	NBPGR3	Exotic	8	8	0	1	3	4
24	AVON1102	Exotic	8	7	1	0	1	7
25	AKON067	Exotic	8	8	0	0	1	7
		Total	208	190	18	6	21	181
		Percent		91.3	8.7	2.9	10.1	87.0

of the specific primers (10 pmol/µl) and volume made up with molecular grade water. PCR amplification for all the markers was done as per Khar and Saini [6]. The amplified PCR products were separated electrophoretically on 2% agarose gel, stained with ethidium bromide, and viewed and photographed under ultraviolet light using the gel documentation system (Cell Biosciences Alphaimager HP).

Suitability of accD in cytoplasm determination

To determine whether primer *acc*D will be able to differentiate N, S, and T cytoplasm, a set of plants with known cytoplasm (N-, S-, and T-) were genotyped. PCR products amplified with *acc*D were sequenced. PCR products were purified using the QIAquick PCR Purification kit (QIAGEN). The purified products were sequenced directly. The sequencing reactions were carried out using BigDye (Applied Biosystems, Foster City, CA, USA) and analyzed using an ABI 3700 Genetic Analyzer (Applied Biosystems) as per the manufacturer's protocol. Differences between the N-, S- and T- cytoplasms were identified by comparing the aligned sequences using the software Clustal Omega [38].

Bulk DNA sampling to test the sensitivity of PCR markers

To reduce the burden of isolating genomic DNA from individual plants for cytoplasm and *Ms* locus determination, the efficiency of a bulking strategy was explored with the assumption that any allele present in a bulk will be detected by the given marker system. Each bulk had a combination of DNA extracted from several individuals from a given group or population. In this study, DNA from already validated male sterile and fertile plants was extracted and pooled in different combinations to assess their effectiveness. Normalized DNA was used for bulking wherein an equal quantity of DNA from individual plants was bulked. DNA from ten plants was pooled in the ratio of 1:9, 2:8, 3:7, 4:6, 5:5, 6:4,

7:3, 8:2, and 9:1. The cytoplasm type and the *Ms* allele genotype were assessed from these DNA pools using the *acc*D and *AcPMS1* primer respectively.

Results

Determination of cytotypes and Ms locus in breeding lines, doubled haploids (DHs), and hybrids

Based on the PCR profile of the marker *acc*D, all the individual plants in the accessions POS13 (14 plants) and POS14 (22 plants) were detected to be S cytoplasmic and N cytoplasmic respectively (Table 1). These same observations were corroborated by the marker MKFR as well. In POS21 and POS22, multiplied as male sterile and maintainer lines, *acc*D and MKFR markers identified all the individual plants as S and N cytoplasm, respectively. Two open-pollinated varieties, Pusa Red (67 plants) and Pusa Madhavi (20 plants), which have been in cultivation for the last 30 years, were also genotyped. In Pusa Red, both *acc*D and MKFR unanimously identified 24 plants with S cytoplasm and 43 plants with N cytoplasm whereas, in Pusa Madhavi, 2 plants with S cytoplasm and 18 plants with N cytoplasm were identified. PMDH2, PMDH3, and PMDH7 are the doubled haploid (DH) lines derived from Pusa Madhavi whereas Hap2hi3.1.2, Haphi3.1.4, Hap2hi3.1.5, Hap2hi3.1.6, Hap2hi3.1.7, and Hap2hi3.1.8 are the DH plants derived from a commercial variety 'Hisar 3'. All the DH lines were identified as N cytoplasmic and both the markers were in agreement. There was no ambiguity in the determination of cytoplasm in DH lines. Three hybrids viz., Juni, Matahari, and T821, commercially available in the market, were also analyzed. In Juni, 100% of the plants had S cytoplasm. In 'Matahari', out of 25 plants, twenty (80%) plants had S cytoplasm whereas five (20%) were having N cytoplasm. Both *acc*D and MKFR gave the same results. In T821, primer *acc*D identified all 47 plants as having N cytoplasm whereas based on MKFR primer, nine (19.1%) plants had S cytoplasm and thirty-eight (80.8%) had T cytoplasm.

Determination of *Ms* locus was carried out using two nuclear markers AcSKP1 and *AcPMS1*. All the plants of POS13 and POS14 carried recessive *msms* locus as determined by these two markers. All the plants of the male-sterile line POS21 and maintainer line POS22 were genotyped as carrying recessive *msms* locus as predicted. The two markers also congruently genotyped the nuclear *Ms* locus of all the plants of the doubled haploid lines derived from Pusa Madhavi as homozygous recessive (*msms*) with 100% efficiency. However, few discrepancies in the determination of the *Ms* locus by these two markers were observed in two of the Hisar-3 derived DH lines viz., Hap2hi3.1.6 and Hap2hi3.1.7. AcSKP1 determined these DH lines as having heterozygous *Msms* genotype whereas primer *AcPMS1* identified them as having recessive *msms* genotype. The rest of the Hisar-3 derived DH lines were found to have recessive *msms* genotype as ascertained by both the nuclear markers. Similar discrepancies between the two markers were observed for Pusa Red and the hybrid lines Juni and Matahari. In Pusa Red, AcSKP1 determined that all the plants had *msms* genotype whereas *AcPMS1* determined sixteen (23.9%) plants having *Msms* genotype and fifty-one plants (76.1%) were having *msms* genotype. In hybrid Juni, all 74 plants were detected to be homozygous recessive at *Ms* by AcSKP1 primer, but according to *AcPMS1* primer, 88% of the plants (66) were homozygous recessive and 12% were heterozygous *Msms*. The results of the AcSKP1 marker in the Matahari hybrid line too revealed 100% plants with homozygous recessive (*msms*) genotype while *AcPMS1* marker determined 72% of the plants with heterozygous (*Msms*) genotype and 28% with the recessive genotype (*msms*).

Determination of cytoplasm and nuclear Ms locus in commercial varieties

PCR markers *acc*D and MKFR were synonymous in cytoplasmic identification. For *Ms* locus genotyping, *AcPMS1* primer was used since this primer predicted more accurate results compared to AcSKP1, based on visual observations. Hence, *acc*D and *AcPMS1* were used for cytoplasm and *Ms* determination in 25 commercially released varieties of Indian onion and some exotic lines grown under short-day conditions (Table 2). Eight individual plants per genotype were used except VL Pyaz where 16 plants were used. Out of 25 accessions, twenty-one accessions had normal 'N' cytoplasm. The remaining four accessions had both N and S cytoplasm. Out of eight plants, Arka Kalyan had three 'N' and five 'S' cytoplasmic plants, Sel. 157 had two normal 'N' and six 'S' cytoplasmic plants, VL Pyaz had ten 'N' and six 'S' cytoplasmic plants whereas AVON1102 had seven normal 'N' and one 'S' cytoplasmic plant. Based on the nuclear *Ms* locus, eighteen accessions had all the plants with '*msms'* constitution. In Arka Kalyan, one plant had '*Msms'* whereas other seven plants were of '*msms'* constitution. In Sel.157, one plant of '*MsMs'*, three of '*Msms',* and four plants of '*msms'* constitution were observed. Similarly, in VL Pyaz, four plants having *MsMs*, ten plants of *Msms,* and two plants of *msms* constitution were identified. In all the four exotic genotypes viz., NBPGR3, AVON1102, NBPGR2, and AKON067, plants of both '*Msms'* and '*msms'* genetic constitution were observed except NBPGR3 where in addition one plant of '*MsMs'* was also observed.

Suitability of accD in cytoplasm determination

Chloroplast marker *accD* can identify S-cytoplasm, but cannot distinguish between the N- and T- cytoplasm. Hence a mitochondrial marker, MKFR was used which could differentiate between N, S, and T cytoplasm. In hybrid T821, primer *acc*D classified all the plants (47) as N cytoplasmic. But MKFR differentiated plants into 9 sterile and 38 T cytoplasmic. To ascertain whether there is any SNPs present between N and T cytoplasm so that this marker (*acc*D) can be used to distinguish between all three cytotypes, PCR amplification of already validated cytotypes (N, S, and T) was done using *acc*D. The PCR product was sequenced directly and sequence similarity was conducted. It was observed that three SNPs and a 45 bp indel sequence were present among S and N and T cytoplasm whereas there was no difference between N and T cytoplasm (Fig. 1).

Sensitivity test of PCR markers

For the determination of cytoplasm and *Ms* locus through bulk strategy, DNA from individual plants having N and S cytoplasm was isolated, normalized, and then mixed in different proportions. Nine pools of 10 plants per sampling group and 9 combination ratios were constituted. This sampling allowed us to evaluate if the molecular markers can efficiently detect the cytoplasm and *Ms* locus in each particular sample size. The optimum sample size was found to be in the ratio of 8S:2 N where the primer *acc*D was able to correctly determine the cytoplasmic nature of the individual plant present in the DNA bulk (Fig. 2). However, the remaining bulk of 9S:1 N was unable to detect N cytoplasm. A similar bulking study was done for determining the usability of bulk samples in male-sterile locus (*Ms*) identification using *AcPMS1* primer. However, the data was inconclusive and only a single band could be observed Fig. (3)

Discussion

Onion is one of the most dominating vegetables in the global food economy along with tomatoes, chilies, and cucurbits [39]. Research and development of hybrid varieties, which are tailored to meet specific requirements like high-yielding or climate and disease resilient, that can match up to the global supply demands takes precedence. In the Indian onion production scenario, despite being one of the leading producers, the research studies on onion hybrids have not gained interest in either public institutions or private enterprises. There may be many reasons for the lack of enthusiasm among the researchers but the main reason is the time required for the identification of male-sterile and maintainer lines through conventional breeding. Further, the wrong selection of a genotype can contaminate the whole population which makes the identification of the maintainer line even more difficult. It is a well-known fact that male sterility in onion involves interaction between the cytoplasm and nuclear *Ms* locus. Through marker-assisted selection, identification of male sterile and maintainer lines in the segregating individuals of onion F1 hybrids has become a reality [16, 24, 40]. During the 1980s, work on male sterility in Indian onion was initiated leading to the identification of male sterile lines and the development of hybrid [6]. Pathak [41] highlighted the problems for hybrid development in India and further hypothesized a new source of male sterility in Indian onion using conventional breeding [42]. After a gap of 20 years, preliminary work on the identification of male sterility was initiated in India and it has gained momentum [30, 31, 43]. Validating the genetic purity of the parental lines and hybrids is indispensable in realizing the full potential of hybrids. So far, there is a lack of concerted effort towards the identification of PCR makers

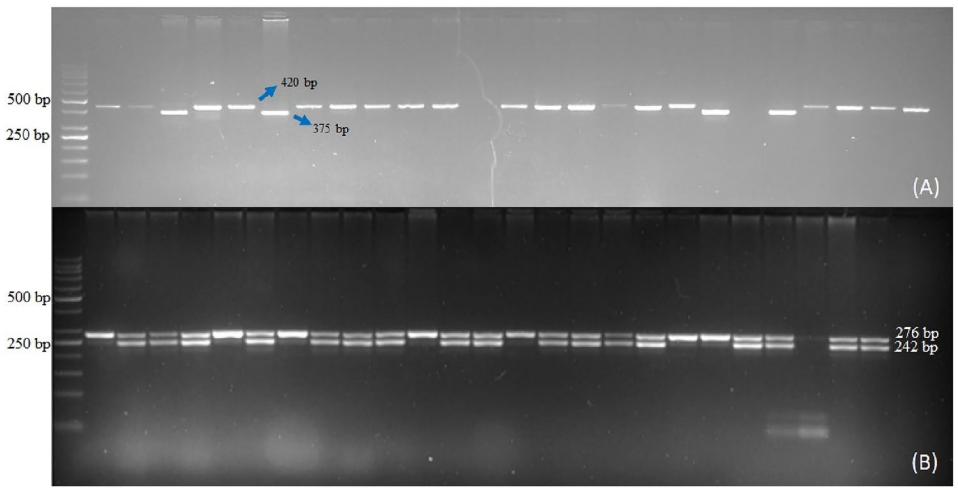

Fig. 1 PCR products of individual plants of Matahari amplified with **A** *acc*D and **B** *AcPMS1* marker

Fig. 2 Sequence similarity of Sterile (S), Normal (N), and T cytoplasm using chloroplast marker *acc*D

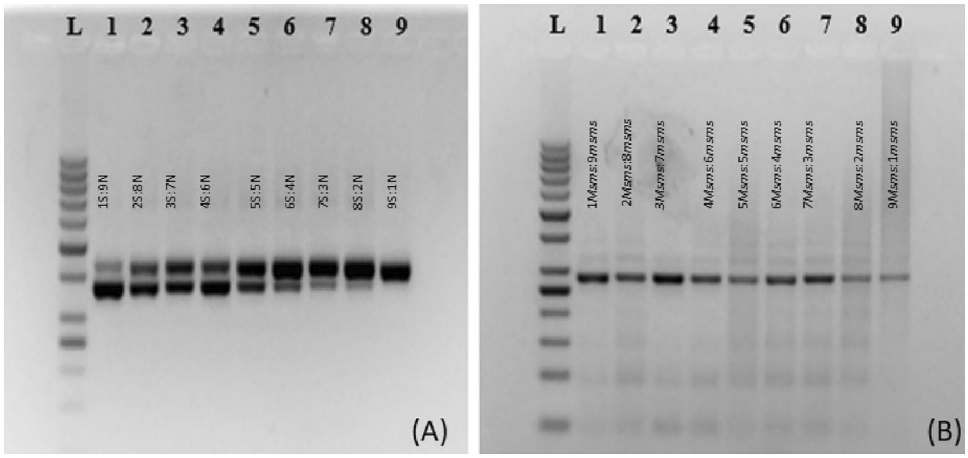

Fig. 3 Amplification of bulked samples using (**A**) *acc*D for cytoplasm and (**B**) *AcPMS1* for *Ms* locus. L- Ladder, S-Sterile cytoplasm; N-Normal cytoplasm

for distinguishing cytoplasm type and determining the *Ms* locus for the development of male sterile, maintainer lines, and consequently hybrid. Hence a step towards this direction was taken up in this study.

In this study, molecular markers that have been developed and validated in previous studies were adopted to determine the cytoplasm type (*acc*D, MKFR) and nuclear genotypes (AcSKP1, *AcPMS1*). PCR markers for cytoplasm were in agreement for most of the onion material used. The only inconsistency was found with hybrid T821. MKFR marker designated 80% of the individual plants as having T cytoplasm whereas *acc*D identified 100% of them as N cytoplasmic. It has been speculated that CMS-N and CMS-T might have emerged from a progenitor (M) cytoplasm as very few polymorphisms have been discovered between their chloroplast and mitochondrial DNA [33, 44, 45]. Holford et al. [44] detected no difference between the chloroplast and the organellar genomes of CMS-T and the fertile N-cytoplasm while studying the origin of male-sterile and male-fertile onions. Havey and Kim [34] reported that mitochondrial *orf725*

marker could not differentiate the inbred lines carrying the N cytoplasm and the bona fide T cytoplasm (proposed to be referred to as R cytoplasm) resulting in the production of only the 833-base pair amplicon. They have proposed that this bonafide CMS-T might be an additional CMS source that is more similar to N and T cytoplasm. Although *acc*D characterized them with N cytoplasm, yet the *orf725* marker produced both the 833 bp and 628 bp amplicons. Further analysis to determine if there is variation between the amplicons produced by the accD marker in genotypes carrying N, S, and T cytoplasm was performed. Sequencing data revealed that there was no difference between the N and T cytoplasm but a 45 bp indel and three SNPs at 208, 223, and 326 bp were observed in between the S, N, and T cytoplasm. This observation gives credence to the observations of Kim and Yoon [46] that CMS-T male sterility has recently developed from normal cytotype. It would be interesting to examine the restoration mechanism in these CMS-T lines to see if the dominant allele of *Ms* locus can restore male fertility. According to Kim [26], male fertility could be restored in male-sterile maternal plants carrying the CMS-T-like cytoplasm when crossed with male-fertile paternal plants as validated by the segregation of male-fertility phenotype and jnurf13 marker genotypes. Havey [9] on the other hand found that male-fertility restoration was not conditioned by the dominant *Ms* allele in CMS-T. He also suggested that the CMS-T-like cytoplasm might have emerged from the Japanese or Dutch population. The mechanism of male-fertility restoration in CMS-S and CMS-T cytoplasmic lines are different. Plants possessing S cytoplasm are restored by a dominant allele at a single locus [8] whereas male fertility restoration in plants with CMS-T cytoplasm is controlled by three independent loci [11]. However, Kim [26] propounded the idea that the three-gene inheritance model proposed for fertility restoration in the CMS-T system may not be the case. Instead, it might be due to the segregation distortion of a single Rf gene. From our study, we suspect the CMS-T plants to be most probably CMS-R (Rijnsburger) cytoplasmic and further examination of the phenotypic and genotypic segregation will allow better determination.

The fertility-restorer (*Ms*) locus was identified using two markers viz., *AcPMS1* and AcSKP1. Male sterile (POS13, POS21) and maintainer line (POS14, POS22) possessed S*msms* and N*msms* genotype, respectively. A similar nuclear genotype was confirmed for Pusa Madhavi-derived DH lines (PMDH2, PMDH3, PMDH7) and Hisar-3 derived DH lines viz. Hap2hi3.1.2, Hap2hi3.1.5, Hap2hi3.1.4, Hap2hi3.1.8) and T821 hybrid. Inconsistency among two markers was observed in Pusa Red, Hap2hi3.1.6, Hap2hi3.1.7, Juni, Matahari, and T821 which brings into question the reliability of these PCR markers for accurate prediction of *Ms* locus. Khar and Saini [6] reported the limitations of PCR markers accurately predicting *Ms* locus. Khrustaleva et al. [32] explained the reason for this phenomenon. The location of *Ms* locus is close to the centromere of chromosome2, a region of relatively low recombination, that gives false positives even though the tightly linked markers are physically distant from *Ms* locus. A thorough prediction of parents on morphological and molecular level followed by selfing and test crossing with the same PCR markers will give a clear picture of the linkage disequilibrium of *Ms* locus in Indian populations. Assessment of cytotypes in Indian varieties and exotic material led to the conclusion that 91.3% of the varieties had N cytoplasm whereas 8.7% had S cytoplasm. S-cytoplasm was present in Indian varieties viz., Arka Kalyan, Sel.157, and VL Pyaz and one exotic accession AVON 1102. Sel. 157 is derived from Early Grano, an exotic variety and VL Pyaz is an exotic introduction whereas the genealogy of Arka Kalyan is not clear. These observations support the hypothesis of Khar and Saini [6] that the source of male sterility in Indian material may be the exotic germplasm. Based on the *AcPMS1* marker, homozygous recessive allele (*msms*) was the predominant *Ms* locus type in the Indian breeding lines and the DH varieties. According to Pike [47], the frequency of plants in a population possessing the N*msms* is less than 5% and a similar observation was made by Pathak [42] who stated that the possibility of the prevalence of germplasm lines involved in crossing program possessing the recessive *ms* gene is very rare. Pathak [42] postulated the possibility of a new source of male sterility in onion. He suggested that although the lines used in his crossing program were possessing dominant *Ms* alleles yet all the F1 hybrids were found to be male sterile. He hypothesized that the resulting sterility was due to a strong cytoplasmic factor. But, based on our observations, it is likely that the *Ms* locus of Indian germplasm used in his crossing programme might have been recessive *ms* alleles instead of assumed dominant alleles. Our data suggest that recessive *msms* alleles are more prevalent in the Indian onion germplasm. In India, mass selection is extensively followed and most of the improved varieties were developed through this method from a local collection of segregating populations. It is highly likely that the varieties that have been used in this selection process possessed recessive *msms* allele that might have been passed on to the germplasm for generations. There are around 45 onion varieties released by the public sector in India and all the varieties have been developed through mass selection. And most of these varieties may track their origin to Central India, Maharashtra, which is the largest grower and supplier of onion in India [48]. This explains the predominance of recessive *Ms* locus.

The suitability of *acc*D primer to differentiate among the N, S, and T cytoplasm was also verified. Chloroplast markers can confidently distinguish N and S cytoplasm [9] but they are unable to identify T cytoplasm. Hence, the use of PCR marker (MKFR) distinguishing S, N, and T cytoplasm

is important in a preliminary assessment of cytoplasms of a particular region. Use of 25–50 plants for DNA isolation [34] is possible in inbreds but in open-pollinated populations, individual plants should be used for DNA isolation. The use of bulk samples for detecting cytotype was correct only when the optimum sample size was 8Sterile:2Normal. But bulk sampling for *Ms* locus was not feasible. We believe this is the first DNA pool study done in onion and this type of study detecting the cytoplasm type from a bulk sample will not only reduce time and resource but can simplify the whole process of selection of the male-sterile, and maintainer line. The DNA pooling analysis study designed here can be adopted for screening numerous plants in a population and then identifying a subset for a more detailed study. The putative identification from the bulk study can be followed up by individual genotyping for identification of the cytoplasm and not for the *Ms* locus.

Conclusion

To conclude, it can be said that both *acc*D and MKFR should be used for the first-time identification of N, S, and T cytotypes in the new accessions. Almost all the onion material grown in India has normal and sterile cytoplasm except T821 which recorded T cytoplasm. To the best of our knowledge, this is the first report of T cytoplasm from India. Research studies to evaluate the effect of maintainer/restorer loci on restoring fertility in this cytoplasm need to be undertaken. More than 95% of the Indian varieties possess homozygous recessive (*msms*) *Ms* locus which can be a good sign for heterosis breeding as the material can be directly used as a maintainer. Limitations of PCR markers to identify *Ms* locus still exist and need to be addressed by utilizing or identifying new PCR markers. The bulking strategy may be a good option for cytotype determination but not for the *Ms* locus. This study is a stepping stone for the heterosis breeding program in Indian short-day onion.

Author contributions AK conceptualized, established and supervised the research study. MCG and AJ provided the facilities for shuttle breeding, bulb development, and field facilities. Bulb planting, DNA extraction, and molecular analysis were performed by MZ, PV and HS. AK and MM performed the data analysis. AK and MZ wrote the paper and organized the manuscript.

Funding This work was financially supported by a research grant awarded under the Core Research Grant-Science and Engineering Research Board (CRG-SERB) Scheme, Government of India (CRG/2019/006525) awarded to the first author.

Data availability All data needed to conduct this study is provided within the manuscript.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors declare that there are no conflicts of interest among them.

Ethical approval Ethical approval was not required because the experiments utilized normal lab conditions only.

Informed consent The study did not use human subjects.

Consent for Publication All authors read the manuscript and showed their willingness to publish this study.

Research involving human and animal participants This work does not contain any studies with human or animal subjects.

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