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Successful deployment of marker assisted selection (MAS) for inbred and hybrid development in long-day onion (*Allium cepa* L.)

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Abstract

In the present investigation, male sterile and maintainer lines were identified using molecular markers in three long day onion populations. Molecular markers, *5'cob* and *orfA501* were able to distinguish effectively normal (N) and sterile (S) cytoplasm in all the three populations. The observed frequency of S cytoplasm in VL *Piaz* 67 (100%), VL *Piaz3* (86.4%) and KR1 (90%) was higher than N cytoplasm. Out of the two PCR markers *viz*., OPT and PsaO used to determine the nuclear fertility restorer locus (*Ms* locus), OPT was found better than PsaO. Increased frequency of dominant homozygous alleles (87.4%) followed by heterozygous alleles (8.6%) and homozygous recessive alleles (4.0%) was observed. Fertility/sterility of plants were validated by acetocarmine staining of the pollens and correlated with observations made using molecular markers. Out of the three populations studied, only 12 plants (2.15%) happened to be completely male sterile in VL *Piaz*-3 and KR1 population, whereas there was no male sterile plant observed among the population in VL *Piaz* 67. Selfing and test crossing of plants having normal (N) cytoplasm led to the development and identification of maintainer lines in *VL Piaz3* and KR1 population. This is the first example of deploying DNA markers for identification and purification of male sterility and hybrid development in long day onion in Indian population.

Key words: Onion, *Allium cepa* L., cytoplasmic male sterility, molecular markers, *Ms* locus

Introduction

Production of hybrid onions became possible with the discovery of male sterile cytoplasm in the onion cultivar 'Italian red' which is conditioned by the sterility inducing cytoplasm (S) and the single nuclear restorer gene in its recessive condition (*ms/ms*) (Jones and Emsweller 1936; Jones and Clarke 1943). Later on, another form of cytoplasm conferring male sterility *i.e*., CMS-(T) was discovered in the onion cultivar 'Jaune paille des Vertus' (Berninger 1965). It was reported that one independent and two complementary genes were involved in fertility restoration of the male sterility (Schweisguth 1973). Of the two systems, CMS-S system is most widely used because of its stability in various environments (Havey 2000). In the CMS-S system, male sterile lines are propagated by crossing with the maintainers possessing N cytoplasm with homozygous recessive alleles at the *Ms* locus (Jones and Davis 1944). Identification of male sterile line on phenotypic basis is relatively easy whereas extraction of maintainer lines from a onion population is difficult and time consuming, due to the biennial nature of onion, high frequency of dominant allele at *Ms* Locus (Havey and Randle 1996) and prevalence of S cytoplasm (Satoh et al. 1993; Havey 1993a; Havey and Bark 1994). Further, developing and evaluating test cross progenies for the homozygosity of the recessive loci (*Ms* locus) with N cytoplasm (maintainers) takes 4-8 years, as onion needs two years (seasons) to complete one cycle. Besides it is time consuming and is not a viable proposition in the present day when advanced non- conventional techniques like availability of tightly linked molecular markers and marker assisted selection (MAS) for onion improvement.

Marker assisted selection (MAS) has a great potential in the genetic enhancement of onion and several breeders expressed the hope to fasten breeding by skipping several breeding cycles during the

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segregating generations and condense timelines (Mazur 1995) and to finally having found a tool to control the allelic variations for all genes of agronomic importance (Peleman and Van Der Voort 2003). Xu and Crouch (2008) opined that the greatest benefits of MAS for the possibilities to achieve the same breeding progress in a much shorter time than through conventional breeding, pyramid combinations of genes that could not be readily combined through other means and to assemble target traits more precisely, with less unintentional losses. Molecular markers distinguishing normal (N) versus sterile (S) cytoplasm (Xu et al. 2008; Sato 1998) and T cytoplasm (Havey 1995; Engelke et al. 2003) have been reported in onion. Hence, identification of molecular markers tightly linked to the nuclear (*Ms*) locus greatly aided the development of maintainer lines. These markers could allow the breeders to identify the plants carrying recessive *ms* alleles and help in identifying the maintainer lines in the first year only. Identification of *ms* allele in dominant, heterozygous and recessive condition have been estimated by employing various types of markers. Gokce and Havey (2002) developed a RFLP based PCR marker to identify the *Ms* locus which was later on converted into PCR marker (Bang et al. 2011; Huo et al. 2012), dominant SCAR makers (Yang et al. 2013), SNP marker (Havey 2013) and codominant PCR markers (Kim 2014) linked to *Ms* locus.

India is the second largest producer and grower of cultivated onion and more than 40 varieties have been released through public sector organizations. Development of hybrids has not caught the attention of breeders because it is difficult to spend 4-6 years for isolating maintainer lines and then developing hybrids. Isolation and maintenance of male steriles and maintainers from the open pollinated varieties/ landraces is an attractive option for the onion breeders to develop hybrids. The present study aimed to validate the available markers for identification and development of male sterile, male fertile (maintainer) and restorers so that hybrid development becomes a viable proposition for the onion breeders. A robust marker aided screening system will help in screening the plants at the seedling level, saving resources and increasing efficiency in the breeding programme.

Material and methods

Plant materials

Three long-day onion populations comprising of commercial variety (VL *Piaz* 3) and breeding populations (KR1 and VL *Piaz* 67) developed for higher productivity were used in this study.

Isolation of DNA, markers and PCR

Total genomic DNA was extracted from leaves using CTAB method given by Saghai-Maroof et al. (1984) with minor modifications. 100 mg tissue was ground in 1 ml extraction buffer [Tris HCl (pH 8.0) (100 mM), EDTA (pH 8.0) (20 mM), NaCl (1.4 M), CTAB (2%), 2-mercaptoethanol (0.4%)] and incubated at 65°C for 30 minutes, cooled at room temperature and centrifuged at 10,000g for 15 minutes. Aqueous phase was extracted by the addition of equal volume of chloroform: isoamyl alcohol (24:1) v/v, incubated for 2 min by slow inversion followed by centrifugation at 10,000g for 15 min. DNA was collected by the addition of iso-propanol to the supernatant, followed by centrifugation at 12,000g for 5 minutes. The DNA pellets were washed twice with 70% (v/v) ethanol, dried and finally dissolved in 50µl Tris buffer containing 1ng/ µl RNAase by incubating at 37°C for 30 minutes. The DNA was diluted to 25ng/µl for further use in PCR.

In order to identify sterile (S) and normal (N) cytoplasm, PCR was performed using the markers anchoring in the upstream region of the mitochondrial gene *cob* (Sato 1998), referred as 5' *cob* markers, and also by primers designed for *orf*A501 gene (Engelke et al. 2003). Two markers linked to putative oligopeptide transporter (OPT) and photosystem I subunit (*Psa*O) reported by Bang et al. (2011) were used to identify fertility restorer gene (*Ms/ms*). The PCR was performed in a 10 µl reaction mixture containing 0.05 µg template DNA, 1 µl 10X PCR buffer, 0.2 µl forward primer (10 µM), 0.2 µl reverse primer (10 µM), 0.2 µl dNTPs (10mM) each and 0.1 µl polymerase mix (Merck, India). PCR amplification of the *5'cob* marker consisted of an initial denaturation step at 94°C for 5 min, 35 cycles at 94°C for 30s, 60°C for 30s and 72°C for 45s and a final 5 min extension at 72°C. The PCR amplification of OPT marker consisted of an initial denaturation step at 94°C for 5 min, 35 cycles at 94°C for 30s, 53°C for 30s and 72°C for 45s and a final extension of 5 min at 72°C. The PCR products were visualized in 1.5% agarose gel after staining with ethidium bromide.

Pollen fertility/sterility test

Viability of the pollens was evaluated when 40-60% flowers of each umbel dehisced to prevent inaccurate evaluation. Flowers were collected in the morning, immediately after anthesis and kept in a closed vessel

with a wet paper towel under high humidity (Lee et al. 1985) to avoid dehydration. Pollen viability was tested within one hour by dispersing pollens in a drop of staining solution (Acetocarmine @ 0.5%) on a microscope slide. Viable pollen stained red whereas non viable pollen remained non-stained or were lightly stained.

Results and discussion

Screening of plants for N and S cytoplasms and fertility restorer gene

Three hundred and twenty five plants (150 from VL *Piaz* 67, 125 from VL *Piaz 3* and 50 from KR1), selected earlier on the basis of morphology (light green anthers) and cytochemical studies (staining of pollens) for fertility/sterility were screened for normal and sterile cytoplasm using PCR-based marker anchoring upstream region of the mitochondrial gene *cob* (*5' cob* marker) and *orfA*501. Both the markers were able to distinguish sterility-inducing 'S' cytoplasm from the normal 'N' cytoplasm (Fig. 1). All plants of VL *Piaz* 67 were found to have sterile cytoplasm whereas in VL *Piaz* 3, 86.4% (108) plants had sterile (S) and 13.6% (17 plants) had normal (N) cytoplasm. Similarly, in the KR1 population 90% plants comprised of sterile (S) cytoplasm whereas 10% were having normal (N) cytoplasm. 5' cob-based marker amplified 180-bp fragment in N cytoplasm whereas in S cytoplasm, amplification of two fragments (180 and 414bp) was observed. Marker *orfA* 501 amplified 473 bp fragment in S cytoplasm and no amplification was observed in N cytoplasm. In a similar study undertaken by Engelke et al. (2003) and Sato (1998), these primers were able to identify the type of onion cytoplasm with a high confidence but the error probability of identifying cytoplasm type is less in case of Sato (1998) because the primers amplify in both S and N cytoplasm. Szklarczyk et al. (2002) also successfully utilized these markers for identification of sterile/normal cytoplasm. During the present study, optimum PCR cycles to obtain good quality band were observed to be 35 whereas Sato et al. (1998) employed 40 cycles for amplification. All the plants derived from VL *Piaz* 67 population were observed to have S cytoplasm and the frequency of S cytoplasm in other two populations was also very high (>85%). Havey (1993b, 1997) could identify open-pollinated populations exclusively possessing S-cytoplasm. The observed frequency of S- cytoplasm in Indian long-day cultivars was more than expected which may be because of the fact that most of the cultivars were derived from the available hybrids and the natural selection did not favour the recessive alleles (*msms*) which led to selection of fertile plants with S cytoplasm. There is a need to further investigate the source of genetic materials released in India through introductions.

All the 325 plants belonging to three populations (VL *Piaz67*, VL *Piaz3* and KR1) were also screened for restorer of fertility (*Ms*) gene using PCR based markers of *OPT* (putative oligopeptide transporter) and *PsaO* (photosystem I subunit O) gene (Bang et al. 2011). Out of 325 plants, 87.4% (284 plants) had dominant homozygous alleles (*MsMs*) for nuclear fertility restorer gene, while 8.6% (28 plants) were heterozygous (*Msms*) and 4% (13 plants) were possessing homozygous recessive (*msms*) allele. All the plants for the populations of VL *Piaz* 67 with only sterile cytoplasm had only dominant homozygous fertility restorer (*MsMs*) alleles. For the populations of VL *Piaz*-3 and KR1, twelve plants were found to be completely male sterile having sterile cytoplasm with homozygous recessive (S *msms*) alleles. In the populations for VL *Piaz* 67, not a single plant was found to be male sterile (S *msms*). Gokce and Havey (2006) developed a model which predicted that there was increase in frequency of dominant (*Ms*) allele in an S cytoplasm population, starting at 0.5 and reaching fixation after approximately 50 generations of random mating whereas the recessive (*ms*) allele exists only in heterozygotes after approximately 15 generations.

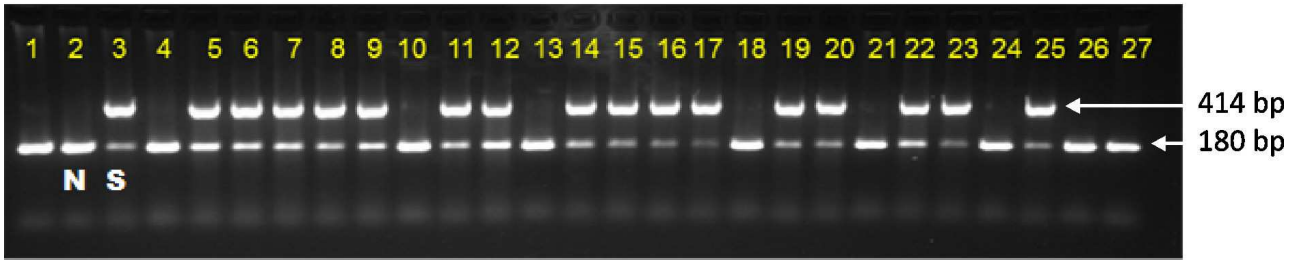

Fig. 1. Polymorphism for 5' *cob* gene differentiating normal (lane-2) and sterile (lane-3) cytoplasm

Changes in Ms allelic frequency towards fixation over relatively short periods of time could have occurred in S cytoplasmic populations such as 'Pukekohe Longkeeper' and 'Creamgold' (Havey 1993b). Extraction of maintainer lines from these onion populations has not been successful due to relatively high frequencies of the dominant *Ms* allele (Havey and Randle 1996; Little et al. 1994; Davis 1957) or S cytoplasm (Satoh et al. 1993; Havey 1993a; Havey and Bark 1994).

Morphological-based evaluation and pollen viability

During the present study, onion flowers were screened morphologically, on the basis of anther colour for pollen sterility. Based on PCR analysis, out of twelve identified sterile plants (S *msms*) seven plants were having light green anthers (Table 1). This reinforces that light green anthers can be an acceptable visual markers for identifying male sterile plants in the initial stage. Pathak (1997) also reported that flowers with light green anthers were possibly male sterile. Although Santos et al. (2010) observed that plants producing flowers with light green anthers were male-sterile but they were not in agreement with the findings of Pathak (1997). During the present studies, all flowers with light green anthers were observed to be male sterile. However all male sterile plants were not observed to have flowers with light green anthers. Hence correlation between the colour of the anthers and male sterility needs further investigation.

The test for viability of pollens was carried out on the selected sterile plants selected on the basis of the PCR analysis. The pollens were stained using acetocarmine stain (0.5%) (Fig. 3a, b). It was observed that the sterile plants had non-viable pollen and were found to be either lightly stained or did not stain at all (colourless) whereas the fertile plants were completely stained and their viability was confirmed. The viability of the pollen completely agreed with the observations made by using molecular markers.

Table 1. Frequency of cytoplasmic and nuclear alleles in onion populations

Population	Plants analyzed	S cytoplasm			N cytoplasm		
		Ms Ms	*Ms ms*	*ms ms*	*Ms Ms*	*Ms ms*	*ms ms*
VL *Piaz* 67	150	150	0	0	0	0	0
VL *Piaz*3	125	93	8	7	12	4	1
KR1 population	50	26	14	5	3	2	0
Total plants	325	269	22	12	15	6	1

Identification of maintainer line

Identification of sterile plants on the basis of colour of anthers is relatively easy in comparison to the identification of maintainer line (N*msms*) where the flowers produce viable pollen and there is no visual marker. A paired test-cross is required to identify the maintainer plant by crossing with sterile plants (Santos et al. 2010). Identification of maintainer line takes at least two years to confirm the plants to be the maintainers. Banga and Petiet (1958) and Costa (1967) took four years to identify maintainer lines by random pairing with male-sterile plants. Selection of one wrong type of plant can contaminate the whole population which makes identification of the maintainer line very difficult. It is now possible to identify/confirm a maintainer line within one year (one season) using marker assisted selection. PCR-based markers, OPT and PsaO, linked to the restorer of fertility (*Ms*) locus (Bang et al. 2011) can also be used to distinguish the *Ms*/*ms* alleles. The OPT marker is very closely located to the *Ms* locus, at a distance of 1.5 cM whereas the marker PsaO is located at the distance of 6.4 cM. During the present study, both the markers were used to distinguish the maintainers. However the OPT marker (Fig. 2) was found to be more effective than the PsaO marker. The PsaO marker predicted homozygous recessive alleles (*msms*) in three out of 48 plants while the OPT marker exhibited the heterozygous state of the alleles which was in full

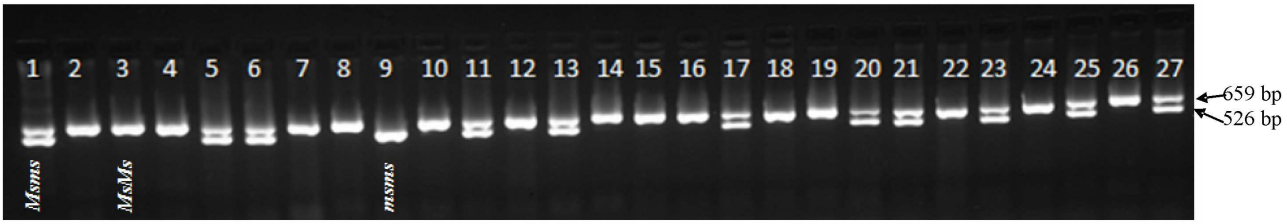

Fig. 2. Polymorphism of nuclear fertility gene; lane 1 (*Msms*), lane 3 (MsMs) and lane 9 (msms)

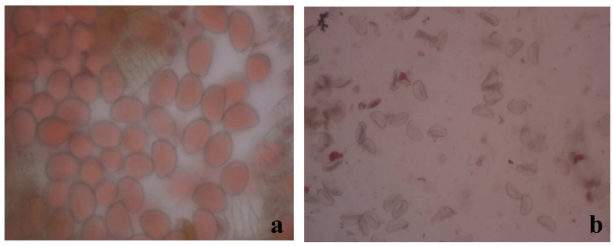

Fig. 3. Staining of pollen grains of onion with acetocarmine (a) viable male fertile pollen (b) non-viable male-sterile pollen

Table 2. Segregation of fertility restorer alleles in selfed progenies of heterozygous plants

Genotype	S cytoplasm		N cytoplasm	
	VL Piaz3	KR1 population	VL Piaz3	KR1 population
MsMs	18	11	9	12
Msms	21	7	14	11
msms	3	2	8	4
Total Plants	42	20	31	27
χ^2 value	10.71	9.90	0.355	5.667
P-Value	0.0047	0.0071	0.8374	0.0588

conformity with the phenotypic observations. Among all the three populations, only one plant in VL*Piaz*3 was found having normal (N) cytoplasm with recessive homozygous (*msms*) gene (Table 1). This plant was treated as a maintainer line and utilized to pollinate the male sterile plants for maintaining the male sterility and selfed to obtain pure seeds of the maintainer lines for use. All the F_1 plants were found to be completely sterile indicating its true maintainer status. In the KR1 population, two plants were found having normal cytoplasm and heterozygous alleles (N *Msms*) (Table 1). Both of these plants were selfed and pollinated with plants having sterile cytoplasm. Resulting F_1 plants and selfed plants were screened to obtain the true A and B type plants. Frequency of homozygous recessive alleles was observed to be approximately 13% (6 out of 47) in the KR 1 population and less than 15% (11 out of 73) in VL *Piaz* 3. In the present study, it was also observed that on selfing of heterozygous plants, the frequency of recessive homozygous allele was quite less than 25% for nuclear fertility restorer gene, particularly in the KR 1 population which need to be further investigated.

The present study could establish the use of molecular markers to distinguish and identify the male sterile (A line) and maintainer line (B line) reliably. Use of linked molecular markers could help in identifying and developing maintainer lines in two years (two seasons) only as compared to at least six years involving the lengthy process of test crosses followed by evaluating progenies for identification of the maintainer lines (Banga and Petiet 1958). These identified 'A' and 'B' lines will be useful for the development of hybrid onion suitable for different parts of India, Asia and elsewhere including long day onions well adapted to the high altitude regions of the North West Himalayan region.

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