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Molecular markers associated with male-sterile cytoplasms and male-fertility restorer locus in onion (*Allium cepa*): a review

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ABSTRACT

Purpose: CMS hybrid seed production systems are employed effectively for onion. Cytoplasmic male sterility (CMS) has long been used to economically produce hybrids that harness growth vigor through heterosis. Three types of onion CMS (CMS-S, CMS-R, and CMS-T) have been used in hybrid onion breeding. **Findings:** The sources of onion CMS, their maintainer plants and fertility restorer lines can be distinguished by markers, saving times spent on crop establishment and avoiding the complex of phenotypic screening. Numerous molecular markers especially PCR markers associated with male-sterile (CMS-S and CMS-T) cytoplasms, male-fertile normal (N) cytoplasm and nuclear-male- fertility restorers (*Ms* locus) were developed. These simple PCR markers are valuable tools for the marker-assisted selection of segregating individuals in onion F1 hybrid breeding programs. The present review reveals practical utility and functional effectiveness in the MAS of male-sterile cytoplasm types with nuclear-fertility-restorer locus. **Limitations:** the environment effect especially temperature may cause male-sterility instability and deviations in segregation ratios for male sterility. Also, maximum exploitation of molecular markers linked to *Ms/ms* and CMS-S, -T genes aid in the recovery of male-sterile traits requires to a perfect linkage disequilibrium that must be investigated further. **Directions for future research:** Furthermore, this information could highly have paved the way for hybrid onion development by applicating of the molecular findings to identify onion male sterility, maintainer and male fertility restorer lines.

INTRODUCTION

F1 hybrids have been used in onion breeding for the past 50 years in the United States, Europe and Japan (Brewster, 2008). Hybrid-onion (*Allium cepa*) seed became economically feasible using sources of CMS. The production of hybrid seed through the CMS system necessitates a male-sterile line (A-line), a male-sterility maintainer line (B-line, male fertile) and male-fertility restorer line (C-, or R-line, male fertile) (Goldman et al., 2002). Male sterility in onion is conditioned by the interaction of the male-sterile cytoplasm with the homozygous recessive genotype at one nuclear locus (*msms*) (A-line) (Yu & Kim, 2021) that all genes responsible for CMS have been existed in mitochondrial genomes (Fauron et al., 1990; Budar et al., 2003; Hanson & Bentolila, 2004; Knoop, 2004). CMS-associated genes often consist on the partial sequences of known mitochondrial genes and unknown sequences (Chen & Liu, 2014; Hanson & Bentolia, 2004; Chen et al., 2017). In most cases, multiple recombination events involving known mitochondrial genes as well as sequences of unknown origin create new open reading frames (ORF) associated with cytoplasmic male sterility in higher plants (Chen & Liu, 2014; Tuteja et al., 2013). These CMS-associated ORFs have been used for producing molecular markers to identify the type of CMS (Khrustaleva et al., 2023). The maintainer line (B-line) of the male-sterile line is produced by crossing normal fertile (N) cytoplasm with the homozygous recessive genotype at one nuclear locus (*msms*). Thus, A- and B-lines are isogenic lines, with the difference only for male sterility trait. Likewise, the male-sterile plants were maintained and reproduced by mating with maintainer plants (Jones & Clarke, 1943; Jones & Davis, 1944). The restorer of fertility (C/R line) governed by single dominant (Ms) allele at the nuclear locus and normal cytoplasm (Manjunathagowda, 2021). This review summarizes the molecular mechanism of male sterility and restoration of male fertility, depicts the identification of male sterility systems through molecular markers that could accelerate breeding of F1 hybrids and production of pure hybrid seed.

CMS cytoplasm

To date, three different types of CMS and fertility restoration systems, have been described and utilized for F1 hybrid cultivar development in onion, termed CMS-S, was first identified during 1925 in the cultivar 'Italian-Red' (Jones & Emsweller, 1936), CMS-R or T-like was identified in 'Rijnsburger' onion (Banga & Petiet, 1958) and CMS-T, traced in the variety 'Jaune paille des Vertus' (Berninger, 1965). The CMS-S is conditioned by the sterility inducing cytoplasm (S) and the single nuclear in its recessive condition (*msms*) with restorer gene *Ms/ms* (Jones & Emsweller, 1936; Jones & Clarke, 1943). The CMS-T is influenced from three independently segregating loci. The male-fertility restoration of CMS-T cytoplasm had been controlled by three Rf genes, one independent gene (a) and two complementary genes (b and c) that revealed a complex nature of inheritance (Schweisguth, 1973).

The discovery of these CMS, paved the way for hybrid onion development, besides uniformity in size, shape, color and maturity and high yields (Khar & Saini, 2016). At present, S cytoplasm due of its stability under diverse environmental conditions and simple monogenic inheritance, is the most common source of CMS used to produce hybrid onion seed and made it more popular among breeders around the world (Havey, 2000). T cytoplasm has been used extensively to produce hybrid onions in European countries, although its use is less common than S cytoplasm (Havey, 1994). The T-cytoplasm had originated from N-cytoplasm through point mutation in the mitochondrial genome. R cytoplasm is very close to N and T cytoplasms and is distantly related to S cytoplasm, and is widely used commercially (Havey & Kim, 2021). In a study with evaluating of onion breeding lines from commercial entities to distinguish sources of onion CMS, their results reveal that T cytoplasm is rarely used

commercially to produce hybrid-onion seed, and both S cytoplasm and "T-like" cytoplasm are widely used (Havey & Kim, 2021). HRM analysis (Khrustaleva et al., 2023) showed that the most and the least source of CMS, was S-cytoplasm and R-cytoplasm, respectively and the proportion of T-cytoplasm among the analyzed onion breeding lines was 20.5%.

Marker assistant selection (MAS) of male sterility, maintainer and restorer lines in onion

Identification of CMS types and *Ms* locus are key steps in onion F1 hybrid breeding. However, since onion is a biennial crop, it takes 4–8 years to identify cytoplasm types and *Ms* alleles using progeny tests (Khrustaleva et al., 2023). Thus, molecular markers can greatly accelerate breeding processes by replacing time-consuming and laborious progeny tests. The conventional method of hybrid development is less efficient as compared to molecular marker assisted methods for identification of S, T, N cytoplasm, maintainer lines and fertility restorer genes with high heterosis. Thus, tightly linked nuclear markers at the *Ms* Locus and CMS genes would allow for molecular-assisted segregation analysis and facilitate the processing of F1 breeding and development. In order to implement reliable marker assisted selection systems for CMS types and the restorer-of-fertility locus (*Ms*) in onions (*Allium cepa* L.), simple PCR based codominant markers linked to the *Ms* Locus were developed (Bang et al., 2011).

Development of polymorphic markers linked to the cytoplasms

Restriction-enzyme analyses of the chloroplast (cpDNA) and mitochondrial (mtDNA) DNAs of normal (N) fertile, S-, and T-cytoplasms have demonstrated fragment size differences (de Courcel et al., 1989; Holford et al., 1991; Havey, 1993; Satoh et al., 1993). de Courcel et al. (1989) delineated four cytoplasmic groups (M I through M4) based on *BamHI* digests of the mtDNA. Restriction enzyme digests of both the mtDNA and cpDNA distinguished S-cytoplasm from the M-cytoplasmic groups (de Courcel et al., 1989). Holford et al. (1991) were able to distinguish among N-, S-, and T-cytoplasms with *BamHI* and *HindIII* digests of mtDNA and among N- and S-cytoplasms with *EcoRI*, *HindIII*, and *XbaI* digests of cpDNA. Havey (1993) after digesting genomic DNA with 15 restriction enzymes and probing with a complete set of chloroplast clones identified five polymorphisms between N- and S-cytoplasms. The complete DNA isolations from single plants, restriction-enzyme digestions, blotting, and hybridizations was time-consuming. For the facilitation of marker-assisted selection and the acceleration of onion hybrids breeding, the Polymerase chain reaction (PCR) would allow a quick and confident identification of the cytoplasm of individual plants. The first published PCR-marker for cytoplasm types in onion amplifies (Havey, 1995) demonstrated that there is a 100-bp insertion in the IGS in (N)-cytoplasm, resulting in a bigger amplicon of corresponding size. Then, further variation within the (M) cytoplasmic group was found and reported for the identification of onion cytotypes (Sato, 1998; Engelke et al., 2003; Kim et al., 2009). Second PCR-marker anchors in the upstream region to the mitochondrial gene *cob* were reported (Sato, 1998). According to the author, the (S)-cytoplasm contains an insertion in this region, which is homologous to the chloroplast *orf1708* of *Nicotiana tabacum*, and which can be used to anchor a (S)-specific primer. Sato (1998) suggested a second primer which should be (N)-specific, and a common antisense primer. Then Engelke et al. (2003) developed a PCR-marker, *orfA501* that distinguished all the three known cytoplasms in the onion, both male sterility inducing cytoplasms, CMS-(S) and CMS-(T), from the normal cytoplasm in onion (*Allium cepa*). The PCR RFLP marker was located in a chloroplast *psbA* gene amplicon could distinguish male-fertile (N) and male-sterile (S) cytoplasm in onions (Cho et al., 2006). Kim et al. (2009) designated a new marker,

orf725. RT-PCR results showed that *orf725* was not transcribed in normal cytoplasm. Meanwhile, the normal *coxI* gene, which is essential for normal mitochondrial function, was not expressed in CMS-S cytoplasm. However, both *orf725* and *coxI* were transcribed in CMS-T cytoplasm. Von Kohn et al. (2013) checked the size of two types of cytoplasm (N and S) and found that the size difference was primarily due to small indels in intergenic regions and a deletion in the *accD* gene of N-cytoplasmic onion. According that result, they designated a new marker, *accD*.

This marker along with *cob* and *MK* were used in detecting of the cytoplasm types of onion Indian germplasm (Khar & Saini, 2016). It was observed that accD should be used more for cytoplasm determination because of the relative simplicity of two primers and its visualization (Khar & Saini, 2016). Dehghani et al. (2021), identified the of *Ms* locus and cytoplasmic types in 123 onion accessions (three populations, male sterile lines and maintainer lines) by three cytoplasmic markers *cob, accD* and *MK* and four nuclear molecular markers (*OPT, PsaO, Jnurf-13 and AcSKP1*). The two markers *accd* and *cob* detected the type of cytoplasm as well, with 100% male sterility for male sterile lines and 100% fertility for maintainer lines. Also, T-type cytoplasm could be distinguished by the *MK* marker.

Development of linked molecular markers to the maintainer lines and the restorer of fertility

In the production of hybrid-onion seed, *Ms/ms* alleles were used in maintainer (B) lines of male sterility (are used to create male-sterile (A) lines) and restorer-of-Fertility (R/C) lines. As a result, identifying restorer (N_{MsMs}) and maintainer (N_{msms}) lines is critical for onion hybrid development (Manjunathagowda, 2021). However, molecular markers capable of distinguishing genotypes at a nuclear locus (*Ms*) are crucial for breeders to save time and effort. Few molecular markers for allelic selection of the nuclear *Ms* Locus have been reported (Gökçe et al., 2002; Martin et al., 2005). For the facilitation of marker-assisted selection, *Ms* locus-specific simple PCR co-dominant markers were created (Bang et al., 2011; Huo et al., 2012; Bang et al., 2013; Kim et al., 2019). Two markers OPT and PSAO were designed by Bang et al. (2011). A simple PCR marker for OPT was developed by designing a primer pairs on the flanking regions of the 108-bp indel which is created by two tandem repeats. The OPT marker was tightly linked to the *Ms* Locus at a distance of 1.5 cM (Bang et al., 2011). Despite the low distance between the OPT marker and Ms locus (1.5 cM), the researcher studies (Khar & Saini, 2016, Dehghani et al., 2021) did not show significant linkage disequilibrium between this marker and the Ms locus, indicating that crossing-over contiguous to the Ms locus mostly occurred throughout the history of onion breeding. Yu and Kim (2021) stated that the discrepancy between genotypes of molecular markers tagging the Ms locus and phenotypes of male-fertility in Indian and Brazilian onion germplasm, is probably because of the presence of the Ms2 locus that needs more studies. Also, it is likely that the relatively low expressivity of the Ms2 locus caused unstable male-sterility (Kim et al., 2019). PSAO marker was designed using tandem repeats (14 and 39 bp) based on the photosystem-I-subunit-O (*PsaO*) gene, which was isolated by genome walking of EST-RFLP probe, which was linked to the *Ms* locus at a istance of 6.5 cM (Bang et al., 2011). A PCR-based marker (WHR240) related to the *AcPME* gene was designed and validated in six different onion lines, and it successfully identified male fertility restorer lines (Huo et al., 2012). Two markers (DNF-566 and RNS-357) from the AFLP markers linked to the *ms* allele might be used to distinguish *MsMs*, *Msms* or *msms* allelic phenotypes among varieties, F1 hybrids and the OP population (Yang et al., 2013). A more reliable simple PCR marker (jnurf13) linked to the *Ms* Locus was constructed using a 12-bp InDel sequence and 5.5 kb flanking sequences. The male-fertility phenotypes of all studied breeding lines were perfectly

matched with marker genotypes (Kim, 2014). Another marker such as ACms.1100 (Bang et al., 2013) and CAPS markers (jnurf05, jnurf06, jnurf10, jnurf17) (Park et al., 2013), having tight linkage with *Ms* Locus, and these markers are of co-dominant nature, thus effectively differentiate the dominant from recessive alleles. The markers linked to cytoplasm types and *Ms* Genotypes and validation of identified markers in different studies are shown in Table 1 and Table 2, respectively.

Table 1. Marker assistant selection of male-sterile cytoplasmic and *Ms* Locus lines for breeding of F_1 hybrid onion.

The Marker based on genes	Method	Cytoplasm's/male-fertility loci	Reference
A two-step HRM marker system with both *cox1* and *orf725* genes	High-resolution melting (HRM) analysis	Identification of the N-, S-, R- and T-cytoplasms	(Khrustaleva et al., 2023)
Ms2 locus was likely to be positioned at the end of chromosome 2	High-resolution melting (HRM) analysis based on single nucleotide polymorphisms (SNPs) detected by RNA-Seq	*Ms* locus	(Yu & kim, 2023)
AcPMS1 gene	PCR	*Ms* locus	(Kim & Kim, 2021)
molecular marker (AcCN) (two markers at the Ms loci and one cytoplasm marker)	multiplex-PCR	CMS and *Ms* locus	(Liu et al., 2019)
100 breeding lines of Onbreetech Corp., Haenam, Korea seed company were employed using CAPS markers	PCR	CMS-S, -T and N-cytoplasm and Ms locus	(Kim & Kim 2015)
301 plants of F2 and F3 populations developed from H6 male-fertile line and 506L male-sterile line using CAPS marker	PCR	Ms locus	(Bang et al., 2013)
	PCR	Ms locus	(Yang et al., 2013)
F2 populations developed from H6 male-fertile line and 506L male- sterile line using OPT and PSAO marker	PCR	Ms locus	(Bang et al., 2011)
176 breeding lines and cultivars using *orf725* marker	PCR	CMS-S, -T and fertile N-cytoplasm	(Kim et al., 2009)
orfA501	PCR	CMS-S, -T and fertile N-cytoplasm	(Engelke et al., 2003)
W202B (*Nmsms)*, W202A (*S msms*) and the S-cytoplasmic male-fertile	PCR	CMS-S and fertile N-cytoplasm	(Sato, 1998)

Table 2. Validation of identified markers in different studies.

Cytoplasm's/male-fertility loci	Genetic material	Reference
cytotype and *Ms* locus	Cytotype markers, *accD*, and *MKFR* and for *Ms* locus identification, PCR markers *AcPMS1* and *AcSKP1* in Indian breeding lines, variety and hybrids	(Khar et al., 2022)
CMS-S, -T and fertile N-cytoplasm/ Ms locus	Three cytoplasmic markers *cob, accD* and *MK* and four nuclear molecular markers (*OPT, PsaO, Jnurf-13 and AcSKP1*) on 123 onion accessions (three populations, male sterile lines and maintainer lines)	(Dehghani et al., 2021)
CMS-S, -T and fertile N-cytoplasm	OPV onion genetic stock of Punjab province using *orf725* markers	(Ahmad et al., 2020)
CMS-S	OPV onion genotypes using *orf725* marker	(Manjunathagowda & Anjanappa, 2020)
CMS Y and *Ms* locus	S1 segregating population was produced from a single plant selected from PI273626 (containing cytotype Y)	(Kim et al., 2019)
CMS-S and -T and fertile N-cytoplasm	Brazilian onion germplasm using *orf725* marker	(Ferreira & Santos, 2018)
CMS-S, -T and fertile N-cytoplasm	5' *cob*/*orfA501* and *orf725* markers deployed in 59 genotypes of the Embrapa Onion Germplasm Bank for MAS of cytoplasms	(Ferreira et al., 2017)
CMS-S, -T and fertile N-cytoplasm/ Ms locus	Five cytoplasmic (5'cob, orfA501, orf725, IGS and cob-type 2) and four nuclear markers (jnurf13, isotig34671_610, isotig30856_1351 and isotig29186_1830)	(Gazendam et al., 2018)
CMS-S and fertile N-cytoplasm	Open-pollinated populations of onion varieties Punjab Naroya, Punjab Selection and Punjab White using '*cob*' marker for cytotype	(Malik et al., 2017)
CMS-S, -T and fertile N-cytoplasm/ Ms locus	Three cytoplasmic markers *cob, accD* and *MK* and four nuclear molecular markers (*OPT, PsaO, Jnurf-13 and AcSKP1*)	(Khar & Saini, 2016)

CONCLUSION

F1 hybrid cultivars are popular due to more bulb uniformity and higher productive potential for heterosis. The sources of onion CMS, their maintainer plants and fertility restorer lines can be distinguished by markers, saving times spent on crop establishment and avoiding the complex of phenotypic screening. Cytoplasmic determinations in onion have been greatly simplified by molecular markers in the mitochondrial and chloroplast DNAs distinguishing N and S cytoplasms (von Kohn et al., 2013). Marker-assisted selection of the PCR markers in determining types of S/N and T cytoplasm and *Ms* locus were reported in Indian onion germplasm (Khar & Saini, 2016) and Brazilian germplasm (Ferreira et al., 2017; Ferreira et al., 2018) and Iranian germplasm (Dehghani et al., 2021). Although these molecular markers do not shorten the generation time of onion, they represent a more sensible use of resources because only plants with known cytoplasms are used for crossing (Havey, 1995). The development of F1 hybrids in onion heterosis breeding was made possible by the use of PCR markers that linked cytoplasms and restorer-of- fertility genes. Many onions in North

America are three-way hybrids. An example is the hybrid 'Spartan Banner 80,' which was developed and released jointly by the USDA and Michigan Agricultural Experiment Station. Another three-way hybrid developed by Dr. Peterson was 'Sweet Sandwich,' from the cross (MSU5718A × MSU8155B) × MSU826B (Havey, 2018).

Two important factors in successful deployment of a CMS breeding pipeline are CMS stability and sterility and fertility transition. Recently, three proposed strategies for the genetic control of sterility and fertility transition to engineer "on–off" switches include gene editing or RNA interference of MSH1, design of Rfs, and mt-targeted gene editing of CMS-associated ORFs in the CMS line (Xu et al., 2022). Recent attempts have been made to develop restorer lines using wide hybridization (Yu et al., 2020) and genetic transformation (Li et al., 2021).

The cp-cytotype RFLP marker (Havey, 1995), cob gene-specific marker (Sato, 1998), orfA501 gene marker (Engelke et al., 2003), the psbA gene marker (Cho et al., 2006), orf725 gene-specific marker (Kim et al., 2009), atp6 gene CAPS marker, atp1 gene, cob gene and cox2 gene-specific markers (Kim & Yoon, 2010), petN gene-specific marker (Kim & Kim, 2015; Kim et al., 2015) and mtDNA CAPS marker (Kim & Kim, 2019) were able to differentiate CMS-S, CMS-T and normal (N) cytoplasm in a mixed population. These cytoplasmic markers, coupled with single nucleotide polymorphisms (SNPs) tightly linked to the nuclear Ms locus (Havey, 2013), can be used to select individual plants using high-throughput genotyping platforms to aid in the development of male-fertile maintainer (N msms) and restorer lines (N/S MsMs) lines for the production of hybrid onion seed (von Kohn et al., 2013). The restorer-of-fertility locus distinguishes through SSCP marker (McCallum et al., 2001), RFLP marker (Gökçe et al., 2002), OPT and PSAO gene-specific markers (Bang et al., 2011), AcPME gene (Huo et al., 2012), CAPS marker (Bang et al., 2013), SCAR marker (Yang et al., 2013), InDel marker (Kim, 2014), AcSKP1 (Huo et al., 2015) and AcPMS1 genes-specific markers (Kim et al., 2015). Liu et al. (2019) designed a multiplex-PCR marker, AcCN that could detect cytoplasm types and the nuclear locus in a single PCR experiment.

Conflict of interest

Author has no conflict of interest, financial or otherwise to declare.

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