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SCREENING OF MOLECULAR MARKER LINKED TO MALE STERILITY IN ONION (*ALLIUM CEPA* L.) GENOTYPES AND ITS VALIDATION

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Abstract

Identification of cytoplasm types, an initial key step in F_1 hybrid breeding takes 4-8 years by progeny testing since onion is a biennial crop. However, molecular markers capable of distinguishing mitotypes at the DNA level enable breeders to save time and effort. An identified molecular marker orf 725 is used in this study to distinguish male sterile and maintainer genotypes maintained at Indian Institute of Horticultural Research Station, Hesaraghatta, Bangalore (Karnataka), India. Among different genotypes tested this marker found to be useful in Arka lalima and Rose onion group to distinguish sterile and maintainer lines at early seedling stages.

Key words: ORF, sterile, maintainer, onion, CMS.

Introduction

Onion (*Allium cepa* L.) is an important commercial vegetable crop, which ranks second in production and cultivation among the vegetables in the world. It is grown for its cuisine and medicinal value. It is one of the oldest cultivated vegetables recorded over 4000 years ago. In India, it is used as a rajasic (having aphrodisiac quality) as reported by Garuda Purana (Shastri, 1995). The medicinal properties of onion and garlic were well known and are documented in Charaka Samhita, an ancient medicinal treatise and also forms an important component of Ayurvedic medicine in modern times (Woodward, 1996 and Van Loon, 2003). Hanelt (1990) proposed that the south west Asia should be acknowledged as the primary centre of domestication and variability of onion, whereas other regions such as the Mediterranean basin is the secondary centre.

On the global front, India ranks second next to China in production of bulb onions. It covers 12.5% of the country's total vegetable area and accounts for 10.3% of the total vegetable production (NHB, 2010). In exports, India stands third after the Netherlands and Spain, contributing foreign exchange earning of over Rs. 1200 crores annually, accounting for 74% of the total vegetable exports from India in terms of value (NHB, 2010). The productivity of onion in India is very low (14.2 t/ha) compared to other countries, *e.g*. USA (53.9 t/ha), Spain (52.1 t/ha) and Japan (47.6 t/ha) (NHB, 2010) due to poor quality seed used by the farmers, use of local varieties and open pollinated varieties. Area under F_1 hybrids is not significant. There is a need to replace OP varieties with F_1 hybrids. Since F_1 hybrids are developed by using male sterile lines, understanding the genetic nature of male sterile lines is most important for development of F_1 hybrids.

The production of hybrid onion (*Allium cepa* L.) seed is economically feasible using cytoplasmic male sterility (CMS) systems. The identification of cytoplasm types might be helpful for development of sterile and maintainer lines. Due to this reason, knowledge of the cytoplasm type is essential for hybrid breeding in onion. Especially, due to the biennial nature, isolation of onion cytoplasm type in individual plant is helpful to reduce the efforts in a breeding program. (Kim- Cheolwoo *et al*., 2007). Molecular markers linked to CMS are useful for propriety claims, for detecting seed mixtures in hybrids, for checking

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contamination of CMS seeds with isogenic maintainer lines, etc.

An open reading frame ORF 725, which was identified in onion mitochondrial genome was responsible for cytoplasmic male sterility in onion. The cytoplasmic male sterile line (S ms/ms) and its isogenic maintainer line (N ms/ms) are essential to breed F_1 hybrids using the CMS system. It takes four to eight years to identify the CMS factor by conventional genetic analysis because onion is a biennial crop. Thus, identifying DNA markers related to CMS is crucial for efficient F_1breeding (Havey, 1995).

Materials and Methods

Plant material

Five genotypes of male sterile (A) their respective maintainers (B) and three hybrids developed using cytoplasmic male sterility were used for marker screening.

Following are the near isogenic lines (NIL's) of onion used in this experiment:

1. MS-65 (A and B line)
2. MS-48 (A and B line)
3. MLT (A and B line)
4. MS-PBR (A and B line)
5. Arka Pragati (A and B line)
6. Arka Kirtiman (MS-65 x Sel-13)
7. Arka lalima (MS-48 x Sel-14)
8. PBR hybrid (MS-65 x PBR)

DNA extraction

Fully developed leaves collected 45 days after transplantation were used for DNA extraction. Total genomic DNA was extracted by the cetyl trimethyl ammonium bromide (CTAB) method described by Havey *et al.* (1996). Briefly, 2 g of leaf tissue were ground to a fine powder in liquid nitrogen and 50 mg polyvinyl pyrollidone (PVPP) was added. The fine powder was transferred to a tube containing 10 ml pre heated extraction buffer [25%(w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 0.5%(v/v) β- mercaptoethanol, 100 mm Tris-Hcl pH 8.0] and incubated for 1 h with occasional shaking. The homogenate was cooled down to room temperature and 10ml Chloroform: Iso amyl alcohol (24:1 v/v) was added. The contents were mixed by inverting the tube gently 25-30 times, and then centrifuged at 5000 x g for 15 min. The clear aqueous phase was transferred to a fresh centrifuge tube and 2.5ml 5M NaCl and 10ml cold ethanol [100%v/v] were added, mixed gently and kept overnight at -20ºC to precipitate the DNA.

Table 1 : Primer sequences used in PCR analysis and molecular marker tests.

Primer names	Primer sequences (5' to 3')
RT-F	TGGGTGGGTAAAATCTTTGGTCGGACA
RT-R1	CACTTTGTCCCACCCGAACTCCAACTC
RT-R2	CCGTTCCGAAGGCGAATAAAAATTTGG

The precipitated DNA was pelleted by centrifuging at 10,000 x g for 10 min at room temperature (25ºC). The supernatant was decanted and the pellet was washed twice with 76% (v/v) ethanol and vacuum dried. The pellet was then dissolved in 1ml Tris-EDTA buffer, pH 8.0 containing pancreatic RNase (20µg ml^{-1}) and incubated at 37ºC for 30 min. The DNA sample was further diluted with 1 ml Tris-EDTA buffer and 1ml 7.5M ammonium acetate added, followed by 10ml cold ethanol [100%v/v] and kept at -20ºC for 1 h. This mixture was centrifuged again at 10,000 x g for 5 min at 4ºC and the supernatant was discarded. The DNA pellet was air-dried at 37ºC and re-suspended in 1ml Tris-EDTA buffer, pH 8.0. The DNA concentration was determined spectrophotometrically at 260nm and its quality verified by electrophoresis in a 0.8% (w/v) agarose gel.

PCR amplification

PCR was performed in a 25µL reaction mixture containing 0.05 µg template, 2.5 µL 10X PCR buffer, 0.5 µL forward primer (10 µM), 0.5 µL reverse primer (10 µM), 5.0 µL dNTPs (10 mM each), and 0.3 µL Taq polymerase mix. PCR amplification was carried out with an initial denaturation step at 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 90 s and a final 10 min extension at 72°C. The primer sequences used in this experiment for PCR and molecular marker tests are presented in table 1.

Validation of male sterility marker

The marker identified was screened on a large population to check its validity by extracting 50 individual plants DNA samples from each line. The floral characters for the same individuals were also recorded in field for back comparison of lab results.

Results and Discussion

The ORF primers (table 1) reported by Kim *et al.* (2009) were screened on different onion male sterile, maintainer and 3 F_1 hybrids which derived through CMS system. It was reported that, with the forward primer RT-F both reverse primers (RT-R1 and RT-R2) will amplify in male sterile genotypes producing two bands of

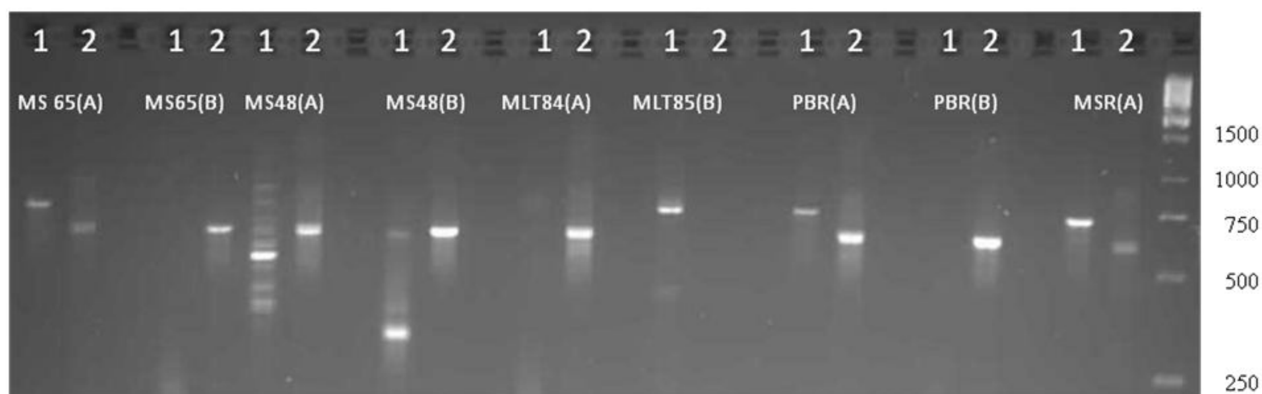

Plate 1a: PCR amplification of orf 725 primers in male sterile and maintainer lines of onion.

1-represents forward primer RT-F with first reverse R1 and 2-represents RT-F with reverse primer R2.

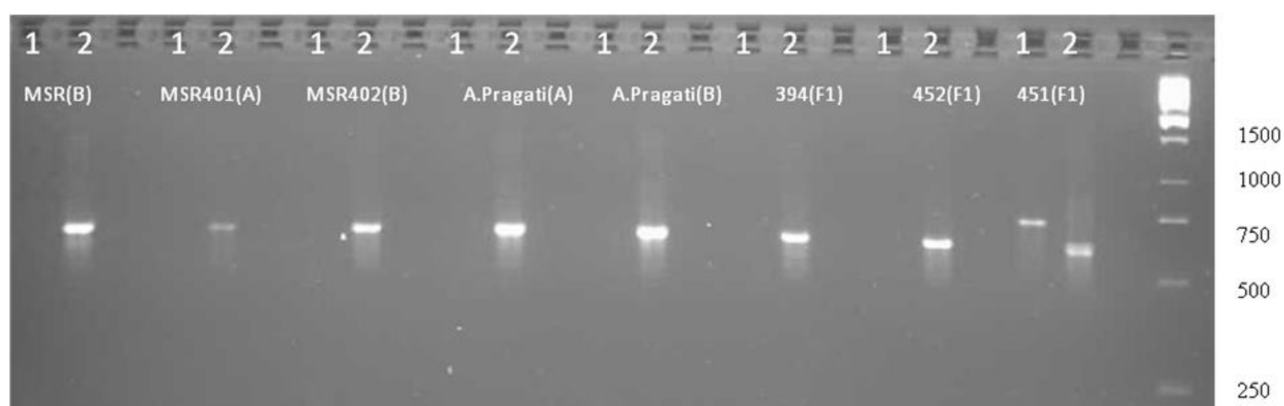

Plate 1b: PCR amplification of orf 725 primers in male sterile, maintainer and hybrids of onion.

1- Represents forward primer RT-F with first reverse R1 and 2-represents RT-F with reverse primer R2.

different molecular weight in two lanes, but in case of maintainer or fertile genotypes only the RT-R2 reverse primer will amplify with the forward primer RT-F to produce a single band.

Among different onion genotypes tested, the lines MS 65A, MSR 486A, MS 174A of Arka lalima, rose onion and PBR group respectively amplified both primers by producing 2 bands of different molecular weight in 2 different lanes, indicating they are male sterile. Their maintainer lines MF 65B, MSR 488B, MF 185B *i.e.*, their B-lines amplified only second reverse primer (RT-R2) with the common forward primer RT-R1 producing single band in the second lane, indicating they are fertile. However among different hybrids tested, which developed through CMS system, only the F_1 hybrid Arka kirtiman (MS-65 x Sel-13) amplified both primers by producing two bands in 2 different lanes indicating its F_1 sterility. The other two hybrids 394(MS65 x PBR), 452(MS48 x Sel-14) and some onion lines MS 48A (447), MS 48B(450), MLT (84A), MLT(85B), MSR(401A), MSR(402B), Arka Pragati A and B line which are suppose to be characterize male sterile and male fertile respectively, amplified only RT-R2 reverse primer producing a single band indicating their instability in maintaining male sterility (plate 1a and 1b). This is because lines are drawn from segregating population. This may be also due to the N-cytoplasmic contamination of S-cytoplasmic hybrid seed lot. Similar results were also reported by Lilly and Havey (2001). So the marker we used is good enough to characterize male sterile and fertile genotypes.

The lines which are proved as male sterile (MS 65A, MSR 486A) and male fertile (MF 65B, MSR 488B) with the ORF marker tests, were subjected to validation. DNA was extracted from individual plants from four genotypes *i.e.*, MS 65A, MF 65B, MSR 486A, MSR 488B (Arka Lalima and Rose onion group) at flowering stage and they were subjected to marker tests. The PCR amplification of 50 individual plants DNA with a set of primers, revealed 100% marker stability by amplifying

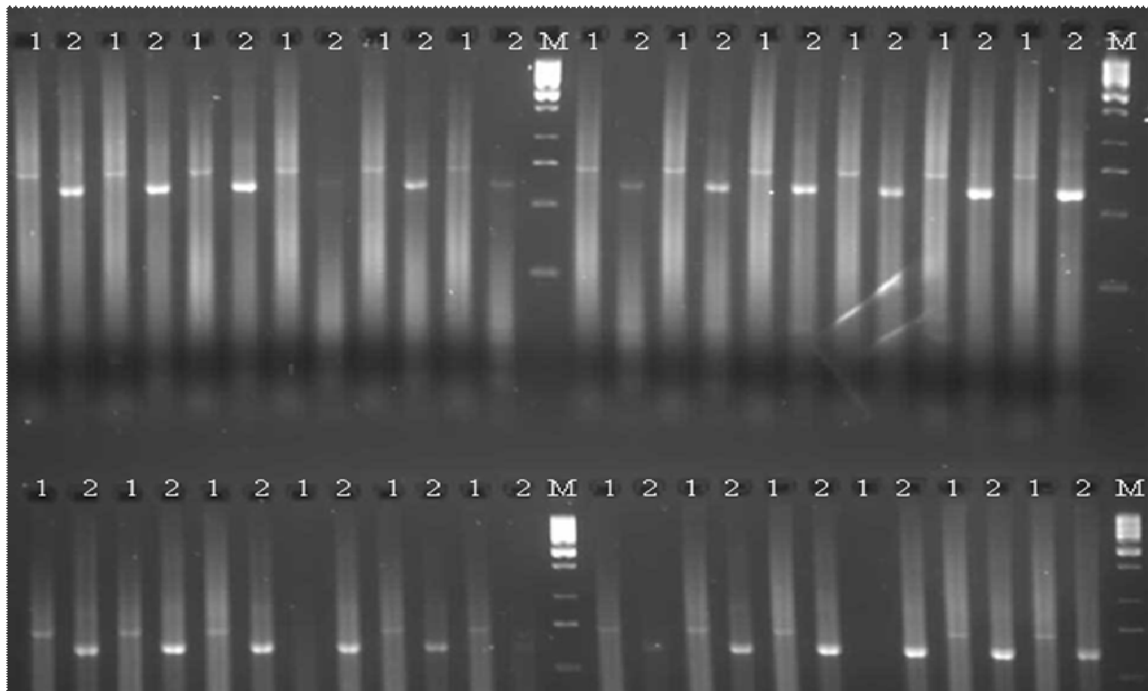

Plate 2a : Validation of MS 65A line showing amplification of both Primers in all the individuals.

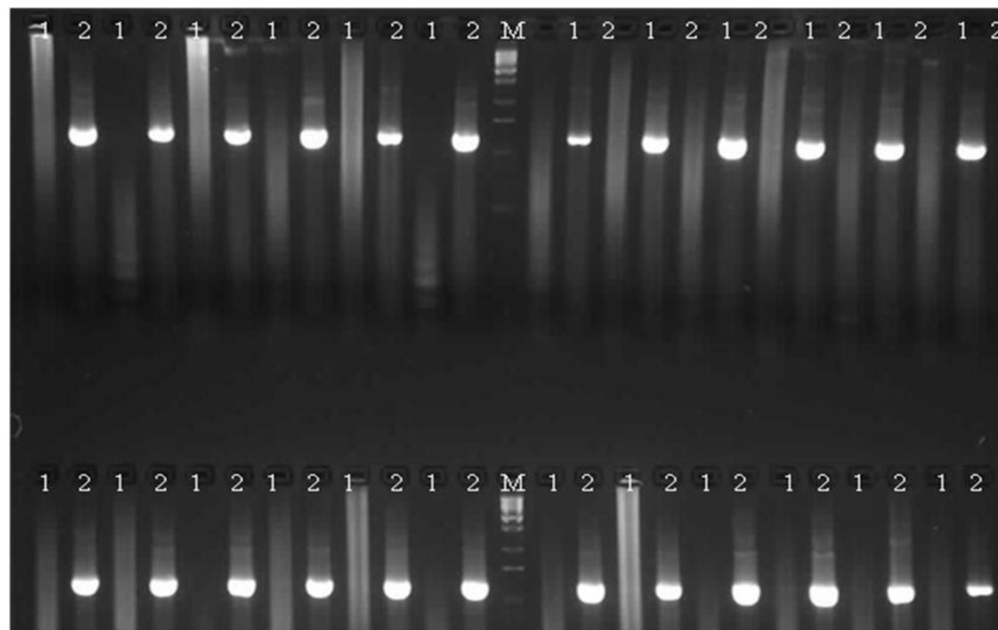

Plate 2b : Validation of MS 65B line showing amplification of second Primer in all the individuals.

both primers producing two bands in all male sterile individuals and amplifying only RT-R2 reverse primer producing single band in all male fertile individuals (plate 2a and 2b). The male sterile lines namely, MS 65A, MSR 486A and their maintainer lines namely, MF 65B, MSR 488B were planted in field for observation of fertility and sterility in lines, also confirmed the results obtained in the lab by genotyping. This indicates the purity of genotypes and also the marker was found to be validating in Arka Lalima and Rose onion group.

Hence, this marker (ORF 725) will allow quick and reliable identification of the cytoplasmic types of individual plants at the seedling stage and assessment of the purity of F_1 seed lots, provided that the F1 s are derived from stable parental lines.

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