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# IDENTIFICATION OF STERILE CYTOPLASM (CMS) IN MAIZE BY USING SPECIFIC mtDNA PRIMERS

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Thirty sources of cytoplasmic male sterility (CMS) from Maize Gene Bank „Zemun Polje, distributed among Yugoslav OP varieties, have been tested for the presence of particular type of cytoplasm by a single seed multiplex PCR approach with specific primer pairs for T, C and S type cytoplasm. Combination of three pairs of primers in a single PCR reaction, corresponding to the chimeric regions of mtDNA sequences specific for each type of CMS, allowed reliable identification of the major CMS types. Dominant presence of S type cytoplasm was detected. For sources where there is no clear identification of the type of cms (absence of the PCR band) there is a reasonable doubt that it could be a new, yet unidentified type of cms.

*Key words*: CMS, maize, multiplex PCR

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## INTRODUCTION

Plant inability to produce functional pollen grains is known as male sterility. Male sterility can be determined by nuclear (genetic male sterility) or cytoplasmic (cytoplasmic male sterility - CMS) genes. CMS has been identified in more than 150 plant species and is successfully used in commercial production of hybrid seed, avoiding the drawbacks of hand or mechanical emasculation (KAUL, 1988).

Three main types of CMS were identified in maize: CMS-T, CMS-S and CMS-C. Male sterile cytoplasm is distinguished by specific nuclear genes (Rf genes) that restore pollen fertility. These genes, called restorers of fertility, suppress the male-sterile effect of the cytoplasm, allowing the production of viable pollen. Commercial production of maize hybrid seed today stands upon utilization of C and S cytoplasmic types. CMS-T was extensively used in 1950-ies and 1960-ies, but showed to be extremely susceptible to *Bipolaris maydis,* pathogen fungi that generates a disease called Southern corn leaf blight (LEVINGS, 1990).

Tester lines containing nuclear Rf genes are traditionally used for identification and classification of CMS types. However, test-crossing procedure is time consuming and labor intensive. Development and appliance of molecular methods revealed that mutations responsible for CMS are located in mitochondrial DNA in many plant species (mtDNA) (SCHNABLE and WISE, 1998). It was confirmed that himeric genes – DNA parts with open reading frames (ORF) that comprise sequences derived from different genes, are responsible for cytoplasmic male sterility. Himeric T-*urf13* gene was detected in mtDNA CMS-T (DEWEY et al., 1986), himeric *atp6-C* gene in mtDNA CMS-C (DEWEY et al., 1991) and a repeated DNA region "R" containing two ORFs in mtDNA CMS-S (ZABALA et al., 1997).

Molecular markers like RFLPs and PCR-based markers that are designed upon the unique characteristics of himeric mtDNA regions can be used for distinguishing the main maize CMS types, much more rapidly than by the traditional test-crossing procedure. RFLP analysis is time consuming to be applied routinely in breeding programs, while PCR-based markers are known to be rapid and definitive identifiers of the cytoplasm (NAKAJIMA et al., 1999; SATO, 1998). Multiplex PCR assay was shown to be a quick and a reliable method and combining three primer pairs in a single reaction makes it convenient for analysis of a huge number of samples (LIU et al., 2002.). This assay reveals 398, 440 and 799 bp specific DNA fragments identifying C, T and S cytoplasm, respectively.

Thirty maize varieties, sources of unknown CMS type from Maize Research Institute Gene bank were subjected to multiplex PCR analysis with specific primers for C, T and S cytoplasm, with the aim to identify their sterility type and check the reliability of the PCR assay compared to the test-crossing procedure.

## MATERIALS AND METHODS

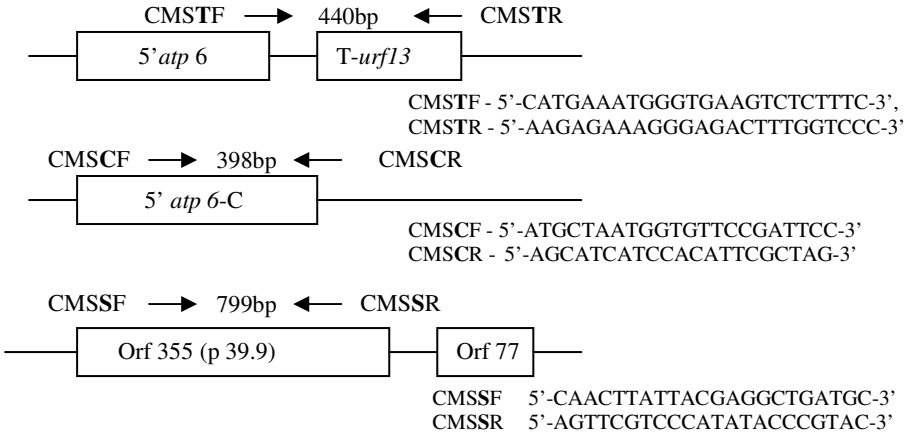
Thirty varieties from Maize Research Institute Gene bank were analyzed. In order to check the reliability of multiplex PCR each variety was analyzed at least three times and the samples were coded. The CMS (T, C and S type) genotypes, as well as one fertile (N type) genotype were used as positive controls, i.e. as markers for identifying the CMS types of the analyzed varieties.

DNA extraction was done from a single seed with CTAB buffer by the modified method of SAGHAI-MAROOF et al. (1984). PCR reaction mix contained 1x PCR buffer, 1.5mM $MgCl_2$, 0.8mM dNTP, 50pmol each primer CMSSF, CMSSR, CMSTF, CMSTR, CMSCF and CMSCR, 1U Taq polymerase, 50ng sample DNA and sterile bidestilated water to the final volume of 25µl/reaction. All three primer pairs specific for T, C and S cytoplasm were synthesized according to the following mtDNA sequences (LIU et al., 2002):

CMSTF 5'-CATGAAATGGGTGAAGTCTCTTTC-3',
CMSTR 5'-AAGAGAAAGGGAGACTTTGGTCCC-3'
CMSCF 5'-ATGCTAATGGTGTTCCGATTCC-3'
CMSCR 5'-AGCATCATCCACATTCGCTAG-3'
CMSSF 5'-CAACTTATTACGAGGCTGATGC-3'
CMSSR 5'-AGTTCGTCCCATATACCCGTAC-3'

The mtDNA recombinant regions of C, S and T sterile cytoplasmic types and the binding sites of the specific primers are presented in Picture 1.

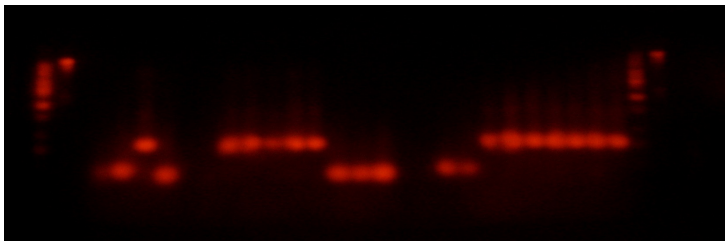
PCR amplification of specific fragments was performed by the following program: 1. $94^0$C/ 2min, 2. $94^0$C/1min, 3. $55^0$C/1min, 4. $72^0$C/1.30min and 5. $72^0$C/5 min. Steps 2 - 4 were repeated in 40 cycles and after the PCR amplified fragments were separated on 1.5% agarose gels and stained with ethidium-bromide.




Picture 1.mtDNA recombinant regions of T, C and S cytoplasm types and the binding sites of the specific primers

## RESULTS AND DISCUSSION

Multiplex PCR analysis of 30 maize varieties from gene bank revealed the dominant (26 genotypes, i.e. 86.6%) presence of S type cytoplasmic male sterility (Table 1). Only one variety was identified as C type (3.3%), while two varieties were identified as T type (6.6%). One variety was identified as a potential source of a new CMS type. In this sample DNA amplification did not occur during the PCR reaction with any of the three specific primer pairs used. Considering that this variety was established as a source of cytoplasmic sterility in the field trials and that no amplification happened with the C, S and T specific primers, it could be assumed that this variety could be a source of a new type of sterility. In order to verify or refute that assumption field trials with restorer tester lines will be conducted. An illustration of electrophoresis of multiplex PCR amplified fragments with specific C, S and T primers is given in Picture 2.



Picture 2 Illustration of multiplex PCR amplified fragments for identification of C, S and T cytoplasm types

Positive controls: A - T type (440bp), B – S type (799bp), C – C type (398bp), D – N typeSamples: 1 – no band, from 2 to 6 – S type, from 7 to 9 – C type, from 10 to 11 no band, from 12 to 13 – T type and from 14 to 20 – S type.

*Table 1 Identified CMS types within 30 varieties from Maize Research Institute Gene bank by single seed multiplex PCR*

| Type of CMS | Number of varieties | % |
|---|---|---|
| CMS-T | 2 | 6.6 |
| CMS-C | 1 | 3.3 |
| CMS-S | 26 | 86.6 |
| No band | 1 | 3.3 |

Although test crossing is the most conclusive method for categorizing maize cytoplasm, different biochemical and molecular protocols have been used with the aim to quicken the analysis. RFLP markers proved to be a reliable tool for

distinguishing the cytoplasm types. A single CMS-C mtDNA probe (pZmCE.510) was reported to reveal the differences among all four cytoplasm types in maize (DEWEY and KORTH, 1994). Unfortunately, RFLP analysis requires highly purified DNA, and at the same time is expensive and time consuming, thus not suitable for routine applications in breeding programs. The multiplex PCR method developed by LIU et al. (2002) uses a combination of six primers in a single reaction and crude seed DNA preparations for the amplification of characteristic mtDNA sequences allowing the reliable identification of the major male sterile cytoplasm types C, S and T. No DNA fragment is amplified from the N-type cytoplasm in this assay because a mtDNA fragment unique for the normal type does not exist. The structural differences between the mtDNA of the sterile cytoplasm types and the mtDNA from normal cytoplasm do not allow the construction of primers amplifying a specific PCR product from the normal cytoplasm.

The multiplex PCR approach showed to be a simple, fast reliable and applicable for large scale screening of maize cytoplasm in breeding programs, reducing time for cytoplasm characterizations from two years to a few days.

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# IDENTIFIKACIJA TIPOVA CITOPLAZMATIČNE STERILNOSTI KUKURUZA PRIMENOM SPECIFIČNIH PRAJMERA mtDNK

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I z v o d

Trideset izvora citoplazmatične muške sterilnosti (CMS) u okviru lokalnih populacija iz Banke gena Instituta za kukuruz “Zemun Polje” je testirano na prisustvo odgovarajućeg tipa citoplazme multipleks PCR metodom, korišćanjem specifičnih prajmera za T, C and S citoplazmu. Kombinovanje tri para prajmera u jednoj PCR reakciji, koji odgovaraju himernim regionima mitohondrijalnih DNK sekvenci specifičnih za svaki tip citoplazme, omogućilo je pouzdanu identifikaciju glavnih tipova sterilene citoplazme. Detektovano je dominantno prisustvo S tipa citoplazme. Za izvore sterilnosti kod kojih nije identifikovan tip sterilne citoplazme (odsustvo PCR trake) postoji realna sumnja da se radi o novim, neidentifikovanim tipovima citoplazmatične muške sterilnosti.

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