UDC 575.633.15
DOI: 10.2298/GENSR1602691S
Original scientific paper

THE APPLICATION OF PROTEIN MARKERS IN CONVERSION OF MAIZE INBRED LINES TO THE CYTOPLASMIC MALE STERILITY BASIS

Milan STEVANOVIC*, Zoran CAMDZIJA, Jovan PAVLOV, Ksenija MARKOVIC, Jelena VANCETOVIC, Snezana MLADENOVIC DRINIC, Milomir FILIPOVIC

Maize Research Institute Zemun Polje, Zemun Polje, Serbia

Stevanovic M., Z. Camdzija, J.Pavlov, K. Markovic, J. Vancetovic, .S. Mladenovic Drinic, M. FIlipovic (2016): *The application of protein markers in conversion of maize inbred lines to the cytoplasmic male sterility basis*.- Genetika vol 48, no2, 691-698.

A total of seven maize inbred lines of different origin and maturity group were used in the trial set up according to the split-plot randomized complete block design in five environments. Each inbred was observed in five variants: original inbred (N); cytoplasmic male sterile C-type (CMS-C); restorer for CMS-C (RfC); cytoplasmic male sterile S-type (CMS-S) and restorer for CMS-S (RfS). The objective was to compare grain yield of original inbreds and their CMS and Rf variants and to apply Isoelectric focusing (IEF) to determine whether the conversion of original inbreds to their CMS and Rf counterparts have been done completely. Protein markers have shown that conversion of almost all inbreds was done good and completely. Only original inbreds ZPL2 and ZPL5 did not concur on banding patterns with their RfC variants. The type of cytoplasm had a very significant impact on grain yield. Namely, CMS-C counterparts significantly out yielded their CMS-S versions, while the inbreds with C and S cytoplasm over yielded inbreds with N cytoplasm, as well as their RfC and RfS versions.

Keywords: banding patterns, grain yield, isoelectric focusing (IEF), male sterility

INTRODUCTION

Maize is one of the most important crops worldwide. It was domesticated in Central America approximately 7700 years ago. It represents a plant species that is evolutionarily most developed in the entire plant kingdom. The revolution in maize production began with the discovery of heterosis and cultivation of the hybrids in USA during the 1930s (DUVICK, 2005). Detasseling of pollen-producing flowers (tassels) on seed-bearing plants in the process of hybrid maize seed production

Corresponding author: Milan Stevanovic,Maize Research Institute Zemun Polje, Slobodana Bajica 1, 11185 Zemun Polje, Serbia ,Phone: +381648406046, Fax: +381113756707 ; e-mail: mstevanovic@mrizp.rs

is a very large-scale operation. Because of that, not only in maize but also in many important field crops, the cytoplasmic male sterility (CMS) trait is used exclusively to produce commercial hybrid seeds (KAESER *et al.*, 2003). Nowadays, production of maize hybrid seed in some parts of the world is increasingly based on using CMS in order to reduce costs of detasseling (STEVANOVIC *et al.*, 2013). CMS is defined as a form of male sterility induced by the complementary action of nuclear and cytoplasmic genes (WEIDER *et al.*, 2009). Specific mutations in mitochondrial DNA (maternally inherited) are responsible for a dysfunction in the respiratory metabolism that can lead to deleterious phenotypes (CILIER *et al.*, 2004; CHASE, 2007), resulting in the abnormal production of male gametes (BUDAR *et al.*, 2003). Therefore, plants either produce no pollen or pollen that is not viable. The majority of studies (STAMP *et al.*, 2000; WEINGARTNER *et al.*, 2002; KAESER *et al.*, 2003) showed a positive effect of CMS on maize grain yield, especially under unfavourable drought conditions (BRUCE *et al.*, 1966). In these cases less nutrients and water are consumed because plants do not form pollen, so instead assimilates are used for the grain filling.

There are three types of CMS known so far in maize (LUO *et al.*, 2002; SHUANGGUI *et al.*, 2006):

1. CMS-T
2. CMS-C
3. CMS-S

The C and S type are used in commercial production in Serbia. CMS-T type is not used due to its specific susceptibility to the fungus *Helminthosporium maydis* race T that caused epiphytotic disease in USA during the 1970s (DUVICK, 1972).

In order to make comparisons of the types of CMS it is necessary to know whether maize inbred lines have been converted to CMS well and completely. There are a few genetic and molecular methods for checking the process of conversion. PCR assay for discrimination of sterile cytoplasm types in maize is one of them (LIU *et al.*, 2002; IGNJATOVIC-MICIC *et al.*, 2006; VANCETOVIC *et al.*, 2010). Isoelectric focusing (IEF) is one of the methods that imply the analysis of proteins in seeds. The application of this method provides determination of genetic purity of maize genotypes based on the electrophoretic separation of alcohol or water-soluble proteins (zeins and albumins, respectively). The IEF method is based on proteins trait that they are stable once they reach their isoelectric point (WESTERMEIER, 2001).

The objective of this study was to compare grain yields of original inbred lines and their CMS and restorer (Rf) variants. Furthermore, the idea was to apply IEF to determine whether the conversion of original inbreds to their CMS and Rf counterparts has been done completely.

MATERIAL AND METHODS

Seven maize inbred lines (designated ZPL1, ZPL2, ZPL3, ZPL4, ZPL5, ZPL6 and ZPL7) of different origin (Lancaster, BSSS and Iodent) and different maturity group (FAO 300-500) were used in the trial. All original inbreds have been developed at the Maize Research Institute Zemun Polje. Each inbred was observed in five variants: original inbred (N), CMS-C, RfC, CMS-S and RfS. Cytoplasmic male sterile and restorer versions have been developed at the Maize Research Institute Zemun Polje converting the inbreds with normal cytoplasm.

The trial, set up according to the randomised split-plot block design, was carried out in three locations in Serbia during two years (2010 and 2011). One location in 2011 was rejected due to a poor crop stand. The three-replication trail was set up in five blocks. Each block represented one type of the studied lines:

I block – N (normal) cytoplasm, i.e. original inbreds
II block – CMS-C inbreds
III block – RfC inbreds
IV block – CMS-S inbreds and
V block – RfS inbreds

Plots within each replication were composed of four rows. Two border rows represented the original inbreds in all five blocks, which was especially important in the CMS blocks, since N inbreds served as pollinators for their sterile counterparts. Plants from two inner rows (avoiding the effect of border hills) were used for the estimation of grain yield. Between spacing was 0.70 m, the elementary plot size amounted to 7.28 m^2, while crop density was 71.429 plants ha^{-1}. The yield was calculated in tones per hectare (t/ha) at grain moisture content of 14%.

Least significant difference (LSD) test was used to compare grain yield of original and converted inbreds at 0.05 and 0.01 levels of significance. The MSTAT-C software package (MSTAT Development Team 1989) was used for performing LSD test. Furthermore, t-tests for grain yield among different variants of the same inbred were performed.

Biochemical characterisation of the original and converted inbred lines was done by the application of ultra thin-layer isoelectric focusing (UTLIEF) of the storage proteins from maize seeds (ISTA, 1999). UTLIEF is an efficient method for both analysing a genetic structure of the germplasm, as well as verification of the maize genotypes.

RESULTS

Of essential importance in hybrid maize seed production based on CMS is that inbreds are completely converted to their CMS and Rf counterparts. Seven protein marker bands, characteristic for each of the observed original maize inbred, were identified analysing the protein profiles by the UTLIEF method. Protein marker bands were selected in accordance to their intensity and resolution on the gel. Figure 1 shows the electropherogram and identified marker bands.

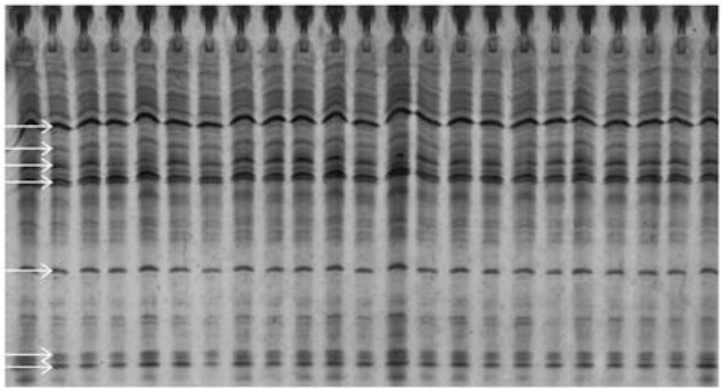

Figure 1. A fraction of the electropherogram of the original inbred ZPL4 with designated protein marker bands

The verification of newly developed genotypes was done according to the presence/absence of protein marker bands, that is, congruence of protein profiles of original inbreds with their counterparts. It was determined that protein profiles of CMS and Rf variants of the inbreds ZPL1, ZPL3, ZPL4, ZPL6 and ZPL7 were identical to original inbreds (Figure 2).

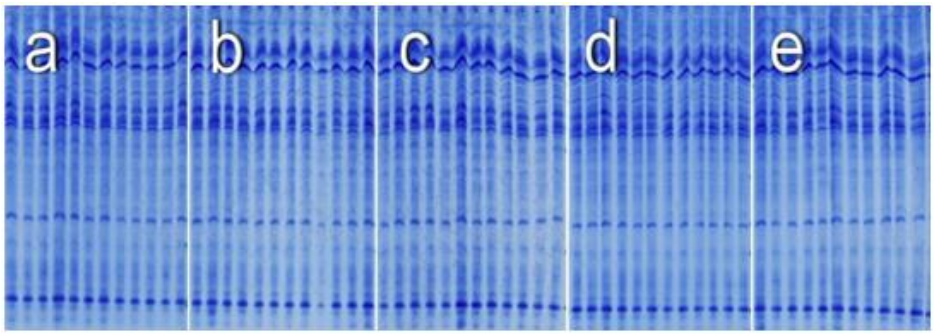

Figure 2. Electropherogram of the original inbred ZPL6 (a) with its CMS-S (b), CMS-C (c), RfS (d) and RfC (e) variants

The comparison of electropherograms of the original inbred ZPL2 with its CMS and Rf variants showed that RfC variant differed according to the presence/absence of protein marker bands from both the original inbred and its remaining CMS and Rf counterparts. The number of marker bands by which this variant differed from remaining ones and the original inbred amounted to eight. On the other hand, the analysis of protein profiles of the inbred ZPL5 showed that its RfC variant differed from remaining CMS and Rf variants and the original line in three protein bands.

The comparisons of grain yield of original and converted inbreds were performed in this study. The estimation of significance of differences between average values was done by LSD test at the 0.05 and 0.01 level (Table 1). The highest yields across all the seven studied inbred lines for both significance levels were detected in variants with C and S cytoplasm (5.303 and 5.197 t ha^{-1}, respectively), while the lowest yield of 4.662 t ha^{-1} was estimated for the variants of RfS type.

Table 1. Least significance difference test for the average grain yield across the five locations

Rank	Type	Value	0.05	0.01
1	CMS-C	5.303	A[1]	A[1]
2	CMS-S	5.197	A	A
3	N	4.878	B	B
4	RfC	4.850	B	B
5	RfS	4.662	C	C
		LSD	0.1283	0.2230

[1] - valued that have no letter in common are statistically different at the given level of significance

Table 2 presents results of t-tests for grain yield among different variants of the same inbred. Only results with statistically significant differences are presented. Grain yield was significantly higher for CMS-C type of the inbred ZPL1 than for the RfS variant of the same inbred. For the inbred ZPL2, the significantly lower yield was detected in the variant with normal cytoplasm than in the RfC and S-type. Moreover, N, CMS-C, RfC and CMS-S versions of the inbred ZPL2 had significantly higher yields than those gained in its RfS version. Differences among grain yields between various versions of the inbred ZPL3 were not established. Significantly higher yields were recorded in CMS-C, CMS-S and RfS versions of the inbred ZPL4 in comparison to the N version, while yields detected in CMS-C and CMS-S types were significantly higher than the yield of the RfC version. For inbred ZPL5 CMS-C and CMS-S variant had significantly higher yield than the original, while CMS-C version was superior both over RfC and RfS variant. Finally, CMS-S variant was superior in comparison with RfC. Additionally, yields recorded in the CMS-C type of the inbred ZPL6 were significantly higher than those detected in N and RfS versions of the same inbred. Grain yield of the CMS-C type of the inbred ZPL7 was significantly higher than yields determined in RfC and RfS versions of the same inbred, while yields recorded for N and CMS-S variants were higher than that recorded for RfC counterpart.

Table 2. t-test for grain yield among different variants of the same inbred line

Inbred	Type	</>	Type	t-test signif.
ZPL1	CMS-C	>	RfS	*
ZPL2	N	<	RfC	*
ZPL2	N	<	CMS-S	*
ZPL2	N	>	RfS	**
ZPL2	CMS-C	>	RfS	*
ZPL2	RfC	>	RfS	**
ZPL2	CMS-S	>	RfS	**
ZPL4	N	<	CMS-C	**
ZPL4	N	<	CMS-S	**
ZPL4	N	<	RfS	*
ZPL4	CMS-C	>	RfC	*
ZPL4	RfC	<	CMS-S	*
ZPL5	N	<	CMS-C	**
ZPL5	N	<	CMS-S	**
ZPL5	CMS-C	>	RfC	**
ZPL5	CMS-C	>	RfS	**
ZPL5	RfC	<	CMS-S	*
ZPL6	N	<	CMS-C	*
ZPL6	CMS-C	>	RfS	**
ZPL7	N	>	RfC	**
ZPL7	CMS-C	>	RfC	**
ZPL7	CMS-C	>	RfS	*
ZPL7	RfC	<	CMS-S	**

*,** - statistically significant at 0.05 and 0.01 level, respectively

DISCUSSION

The application of isoelectric focusing has not yet been used for the verification of conversion of maize original lines to CMS and/or Rf inbreds, hence the use of these markers in future could be used as a rapid and efficient tool to compare original and converted inbreds. Therefore, these markers can provide information on whether the conversion process has been done well and completely and whether out-pollination of converted inbreds has been occurred. Furthermore, the DNA technique based on PCR is characterized by high cost and a complex experimental procedure, which is currently hard to apply in a large-scale and commercial seed quality testing. According to the results obtained by use of protein markers, the conversion process of almost all inbreds in our research has been done completely. Only results that did not concur on banding patterns were those obtained for original inbreds and inbreds ZPL2 RfC and ZPL5 RfC. It can be stated that these two inbreds have not been converted completely and that some more backcrosses are necessary to complete their conversion. Additionally, ZPL2 RfC variant had significantly higher grain yield than its original version, that can be due to the residual heterozygosis of the RfC version detected by IEF. On the other hand, this was not the case with ZPL5.

The effect of CMS on grain yield is expected to be positive due to lower consumption of energy and nutrients caused by the absence of pollen formation. In our research inbreds with C and S type cytoplasm over yielded inbreds with N cytoplasm, as well as RfC and RfS versions. Our results are in accordance with the results of previous studies conducted with T, S and C type cytoplasm (BRUCE *et al,.* 1966; DUVICK (1972)). More recent studies conducted by STAMP *et al.*, (2000) on newly developed hybrids showed significant positive effects of CMS on grain yield under different environmental conditions. WEINGARTNER *et al*., (2002) and KAESER *et al*., (2003), also proved a positive effect of CMS on grain yield. On the other hand, investigation of BOZINOVIC *et al.* (2015) did not show positive effect of CMS on grain yield. MUNSCH *et al.* (2009) determined both positive and negative CMS effects on grain yield.

In all the five environment in our research neither inbred line showed the late break of sterility nor in the CMS-C nor in the CMS-S type. On the other hand, WEIDER *et al.,* (2009) in a comprehensive study reported the highest stability of CMS-T type across 17 environments in two years of testing. CMS-S type exposed the least stability in their investigation.

The comparison of C and S type with N-type showed that these two types of sterility positively affected grain yield. The comparison of C type with S type indicated that C type was superior in grain yield. Due to this, it can be concluded that C type is somewhat more suitable for seed production, which has been confirmed by previous studies of DUVICK (1972), COCHRAN (1975) and JOSEPHSON *et al.*, (1978). They have showed that CMS-C type was more suitable for hybrid seed production than CMS-S type, but less suitable than CMS-T type. A lot of significant differences in grain yield among different variants of the same inbred proven by the t-tests in our research pointed out to pleiotropic effects of CMS and/or Rf genes or to their interactions with the environment.

It is observable that many studies on CMS effects on grain yield were conducted until 1970s. Subsequently, studies within this field have been slack, because the T-type cytoplasm have expressed its intolerance to pathogens and diseases. Not earlier than 2000 had we any information on CMS effects on hybrid yield (STAMP *et al.,* 2000). In recent decades, genetics has provided significant improvements in relation to genetic potential of grain yield in maize (RUSSELL, 1991; DUVICK, 2005). Therefore, the CMS effects on grain yield of new modern hybrids should be re-

examined. Results of the present study indicate that inbreds with C type cytoplasm over yielded remaining variants, including inbreds with S type cytoplasm, so C-type would be preferable for hybrid seed production of modern maize hybrids.

Received January 11th, 2016
Accepted May25th, 2016

REFERENCES

BOZINOVIC S., S. PRODANOVIC, J. VANCETOVIC, A. NIKOLIC, D. RISTIC, M. KOSTADINOVIC and D. IGNJATOVIC-MICIC (2015): Individual and combined (Plus-hybrid) effect of cytoplasmic male sterility and xenia on maize grain yield. Chilean J. Agri. Res., *75*: 160-167.

BRUCE R. R., J. O. SANFORD and D. L. MYHRE (1966): Soil water and nitrogen influence on growth and fruiting of a cytoplasmic male-sterile corn hybrid and its fertile counterpart. Agron. J., *58*: 631-634.

BUDAR F., P. TOUZET and R. DE PAEPE (2003): The nucleo-mitochondrial conflict in cytoplasmic male sterilities revisited. Genetics, *117*: 3–16.

CHASE C. D. (2007): Cytoplasmic male sterility: A window to the world of plant mitochondrial–nuclear interactions. Trends Genet., *23*: 81–90.

CILIER M., D. FERUZAN and O. GÖKSEL (2004): Histological aspects of anther wall in male fertile and cytoplasmic male sterile *Helianthus annuus* L. Asian J. Plant Sci., *3*: 145–150.

COCHRAN D. E. (1975): Progress of cytoplasmic and genetic sterility in hybrid seed corn production. Proc. of the 30th Ann. Corn and Sorghum Res. Conf., *30*: 116-130.

DUVICK D. N. (1972): Potential usfullness of new cytoplasmic male steriles and sterility systems. Proc. of the 27th Ann. Corn and Sorghum Res. Conf., *27*: 197-201.

DUVICK D. N. (2005): Genetic progress in yield of United States maize (*Zea mays* L.). Maydica, *50*: 193-202.

IGNJATOVIC-MICIC D., A. NIKOLIC, S. MLADENOVIC DRINIC, J. VANCETOVIC and V. LAZIC-JANCIC (2006): Identification of sterile cytoplasm (CMS) in maize using specific mtDNA primers. Genetika, *38*: 227-233.

ISTA (International Seed Testing Association) - (1999): International rules for seed testing. Annexes: 1999. Seed Sci. Technol.. *27* (supplement).

JOSEPHSON L. M., T. E. MORGAN and J. M. ARNOLD (1978): Genetics and inheritance of fertility restoration of male-sterile cytoplasms in corn. Proc. of the 33rd Ann. Corn Sorghum Res. Conf., *33*, 48-59.

KAESER O., U. WEINGARTNER, K.-H. CAMP, S. CHOWCHONG and P. STAMP (2003): Impact of different CMS types on grain yield of dent × flint hybrids of maize (*Zea mays* L.). Maydica, *48*: 15-20.

LIU Z., O. S. PETER, M. LONG, U. WEINGARTNER, P. STAMP and O. KAESER (2002): A PCR assay for rapid discrimination of sterile cytoplasm types in maize. Crop Sci., *42*: 566-569.

LUO H., H. HUANG, M. LONG, W. ZHOU, X. LIU and Y. YANG (2002): Responses of cytoplasmic male sterile lines to various restoring resources. Maydica, *47*: 67-69.

MSTAT Development Team, (1989): MSTAT-C: A microcomputer program for the design, management and analysis of agronomic research experiments. MSTAT Development Team, Michigan State University, East Lansing, USA.

MUNSCH M., K. H. CAMP, P. STAMP and C. WEIDER (2009): Modern maize hybrids can improve grain yield as plus-hybrids by the combined effect of cytoplasmic male sterility and allo pollination. Maydica, *53*: 261-268.

RUSSELL W. A. (1991): Genetic improvement of maize yields. Adv. Agron., *46*: 245-298.

SHUANGGUI T., X. JUNHONG, Q. FAZHAN and Z. YONGLIAN (2006): Genome-wide analysis of maize cytoplasmic male sterility-S based on QTL mapping. Plant Mol. Biology Rep., *24*: 71-80.

STAMP P., S. CHOWCHONG, M. MENZI, U. WEINGARTNER and O. KAESER (2000): Increase in the yield of cytoplasmic male sterile maize revisited. Crop Sci., *40*: 1586-1587.

STEVANOVIC M., Z. CAMDZIJA, J. PAVLOV, M. CREVAR, N. GRCIC, G. STANKOVIC and S. MLADENOVIC DRINIC (2013): The effect of cytoplasmic male sterility on_yield stability of maize inbred lines. IV International symposium "Agrosym 2013", October 3-6, Jahorina, Bosnia and Herzegovina. Conference book, pp. 95-100.

VANCETOVIC J., M. VIDAKOVIC, D. IGNJATOVIC-MICIC, A. NIKOLIC, K. MARKOVIC and V. ANDJELKOVIC (2010): The structure of sterile cytoplasm types within a maize genebank collection. Russ. J. Genetics, *44*: 1317-1324.

WEIDER C., P. STAMP, N. CHRISTOV, A. HUSKEN, X. FOUEILLASSAR, K.-H. CAMP and M. MUNSCH (2009): Stability of cytoplasmic male sterility in maize under different environmental conditions. Crop Sci., *49*: 77-84.

WEINGARTNER U., O. KAESER, M. LONG and P. STAMP (2002): Combining cytoplasmic male sterility and xenia increases grain yield of maize hybrids. Crop Sci., *42*: 127-134.

WESTERMEIER R. (2001): Electrophoresis in Practice, 3rd Ed. Wiley-VCH, Weinheim, Germany.

PRIMENA PROTEINSKIH MARKERA U KONVERZIJI INBRED LINIJA KUKUURZA NA BAZI CITOPLAZMATIČNE MUŠKE STERILNOSTI

Milan STEVANOVIC*, Zoran CAMDZIJA, Jovan PAVLOV, Ksenija MARKOVIC, Jelena VANCETOVIC, Snezana MLADENOVIC DRINIC, Milomir FILIPOVIC

Institut za kukuruz Zemun Polje, Zemun Polje, Beograd, Srbija

Izvod

U ovom radu ispitivano je sedam inbred linija kukuruza različitog porekla i dužine vegetacije (FAO 300-500). Svaka od linija je ispitivana u pet varijanti:

1. Originalna linija (N)
2. CMS-C
3. Rf-C
4. CMS-S, i
5. Rf-S

Prevođenje na sterilne i restorer verzije linija je vršeno u Institutu za kukuruz "Zemun Polje" konverzijom linija sa normalnom citoplazmom. Cilj ovog istraživanja je bio poređenje prinosa zrna originalnih linija i njihovih CMS i Rf varijanti. Nadalje, ideja je bila da se primeni tehnika IEF (Izoelektričnog fokusiranja) kako bi se ustanovilo da li je prevođenje originalnih linija urađeno kako treba i do kraja. Na osnovu rezultata dobijenih čitanjem proteinskih traka ustanovljeno je da je proces prevođenja skoro svih linija urađen kako treba. Jedino prevođenje originalnih linija ZPL2 i ZPL5 nije do kraja urađeno na njihove RfC verzije. Važno je napomenuti i to da je tip citoplazme imao veoma značajan uticaj na prinos zrna. Pokazalo se da linije sa C i S tipom citoplazme daju viši prinos nego njihovi N, RfC i RfS analozi.

Primljeno 11. I. 2016
Odobreno 25. V..2016.