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The use of DNA markers for the evaluation of maize lines and hybrids based on cytoplasmic male sterility

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Abstract. The use of cytoplasmic male sterility (CMS) is very important for the production of maize hybrids. The new inbred lines and hybrids of maize of Ukrainian breeding were studied. In the field, four pairs of sterile analogue of line RL106S were obtained during four backcrossing (17M, 19M, 23M, 27M and 29M) and maintainer lines of RL106fS (18M, 20M, 24M, 28M and 30M) for S type cytoplasm and RL108C (195C, 201C, 205C, 207C, 209C) i RL108fC (196C, 202C, 206C, 208C, 210C) for C type respectively. For S type, the following combinations were obtained: RL23S×RL106fS, RL107S×RL106fS, RL98S×RL106fS, RL105S×RL106fS, RL113S×RL106fS and for C type: RL109C×RL108fC, RL110C×RL108fC, RL112C×RL108fC, RL114C×RL108fC, RL115C×RL108fC. The obtained hybrid combinations were planted the following year in a control nursery for field trials. According to the results of the field assessment, all the hybrids were sterile. The types of sterility of the studied lines and hybrids were determined using polymerase chain reaction (PCR) with specific primers for C and S types of cytoplasms. The presence of specific amplicons 398 and 799 bp was determined in sterile lines with C and S types of cytoplasm, respectively. Amplicons 398 and 799 bp were identified in simple-cross and simple reconstituted hybrids on a sterile basis, and can be used to determine the type of hybrid and its maternal component at the stages of selection and examination of new hybrids.

Key words: *Zea mays* L., DNA markers, cytoplasmic sterility.

INTRODUCTION

The traditional crop for biogas production and alternative energy source in the world is maize on silage (Križan et al., 2017; Križan et al., 2018). In comparison to sunflower and straw, maize has better fusibility, lower content of sulfur and chlorine (Lisowski et al., 2019). Many factors and specific features are taken into account during the growing of maize on silage: selection of hybrids of different maturity group, time and methods of sowing, density of planting, harvesting time. For each soil and climatic zone, hybrids of different biotypes are selected, which are capable of forming not only a high green herbage yield, but also a considerable part of the grain of middle dough stage. Parent components are important in the production of hybrids (Gayosso-Barragán et al., 2020). The use of cytoplasmic male sterility (CMS) enables to reduce the cost for cutting of tassels of maternity plant significantly (Allen et al., 2007; Chekanova, 2013). Three types of maize CMS are most widely: T type (Texas), S type (USDA) and C type (Charrua). The

use of T type has been greatly reduced because it confers susceptibility to *Helminthosporium maydis*, thus types S and C are prevalent in breeding and in the maize seed industry. CMS is expressed when a sterility factor is present in the cytoplasm and recessive alleles of the restoration of fertility (Rf) genes are located in the nucleus (Laughnan & Gabay-Laughnan, 1983; Konovalov et al., 1990; Krivosheev & Ignatev, 2019). Tester lines containing nuclear Rf genes are traditionally used to determine and classify the CMS types (Liu et al., 2002; Anisimova & Gavrilova, 2012). However, the test-crossing procedure is a laborious process which requires a lot of time and material resources.

The determination of the sterility of maize lines is a part of the qualification examination of plant varieties for distinctness, uniformity and stability (DUS). The guide of DUS includes the determination time of anthesis and anthocyanin colouration at base of glume of tassel, which can be used to find distinctness between lines and their sterile analogues. However, it is known that the ability to form anthers of maize may depend on environmental factors, as well as the time of observation. These reasons are connected with additional difficulties for an objective assessment of lines during qualification examination. Thus, it is relevant to use fast and reliable methods for identifying the main types of sterility in seeds and in vegetative plants. The knowledge of molecular structures and mechanisms that underlie of CMS has increased significantly with the development of molecular genetic analysis methods. It is known that mutations responsible for CMS reside in mitochondrial DNA (mtDNA) in many plant species (Liu et al., 2002; Palilova et al., 2005). The results of a study of the mitochondrial genome structure and gene expression have enabled to use them for determination the major types of maize cytoplasm.

The polymorphism of mtDNA can be assessed by molecular genetic markers (Manson et al., 1986; Stevanovic et al., 2016). The application of RFLP (Restriction Fragment Length Polymorphism) analysis and methods based on polymerase chain reaction (PCR) are described. Specific primers were developed by Liu et al. (2002) to identify the three major types of CMS based on the mtDNA sequence. The studies were conducted to evaluate the molecular genetic polymorphism of mtDNA in maize and to determine new subtypes of S sterility in maize using DNA markers (Slischuk et al, 2011; Vancetovic et al., 2013). Thus, the aim of this research is to identify the C and S types of sterility in maize by DNA markers for using in DUS examination of varieties.

MATERIALS AND METHODS

Plant material

The lines and hybrids of maize were provided by Research Institute of Agrarian Business (Dnipro, Ukraine). Plant material of maize associated with S and C sterility types included: 5 pairs of sterile analogues of lines and their maintainers, 5 sterile analogues of lines, 5 sterility based single-cross hybrids and 2 restored hybrids.

The sterile analogues of lines were obtained by the backcrossing method (Mustyatsa & Mistrets, 2007) for 5–7 cycles. The main requirements to sterile analogues were the complete sterility of plants and selection within the line for the ability to maintain sterility. As it is known, almost all lines of heterotic plasma Iodent have ability to restore C type of CMS that is why they are used as maternal forms of hybrids which is possible based only on S type of CMS. To study the C type of CMS, the Lancaster heterotic group lines were used (Table 1).

Table 1. Characteristics of S and C type of maize CMS lines

Line	S type	Heterotic group	Line	C type	Heterotic group
RL106S	sterile	Iodent	RL108C	sterile	Lancaster
RL106fS	fertile	Iodent	RL108fC	fertile	Lancaster
RL23S	sterile	Iodent	RL109C	sterile	Lancaster
RL107S	sterile	Iodent	RL110C	sterile	Lancaster
RL98S	sterile	Iodent	RL112C	sterile	Lancaster
RL105S	sterile	Iodent	RL114C	sterile	Lancaster
RL113S	sterile	Iodent	RL115C	sterile	Lancaster

The level of tassels sterility during flowering was determined by examining them in the breeding nursery. According to the results of the evaluation, the studied samples were grouped by sterility classes (Gontarovskiy, 1971): class 0 – complete sterility, all or almost all sterile anthers are in closed spikelets; class 1 – complete sterility, a significant amount of sterile anthers emerge; class 2 – incomplete sterility, the number of fertile anthers does not exceed 25%; class 3 – partial fertility, the number of fertile anthers is 25–75%; class 4 – incomplete fertility, the number of fertile anthers > 75%, sterile anthers are rare; class 5 – complete fertility.

Field studies were carried out in pilot plots of Research Institute of Agrarian Business (Vesele village, Dnipropetrovsk region, Ukraine), laboratory studies were conducted in Laboratory Molecular Genetic Analysis of Ukrainian Institute for Plant Variety Examination (Kyiv, Ukraine) during 2018–2019.

DNA extraction and PCR

DNA was isolated from 50 mg of green maize leaves in five repetitions (leaves from five separate plants of each sample). The extraction approach includes the use of CTAB as a lysing solution, double purification the mixture with chloroform and dissolution DNA in TE buffer (solution with Tris and EDTA) (Velikov, 2013; Aukenov et al., 2014; Gupta, 2019; Prysiazhniuk et al., 2019). Two pairs of primers to mitochondrial genes were used in the study according to two main types of sterility (Liu et al., 2002). The sequences and characteristics of the primers are shown in Table 2.

Table 2. Primer characteristics

Type of cms	Sequences 5'→3'	Amplicons size, bp	Sequence GenBank
CMS C	F – ATGCTAATGGTGTTCCGATTCC R – AGCATCATCCACATTCGCTAG	398	S81074
CMS S	F – CAACTTATTACGAGGCTGATGC R – AGTTCGTCCCATATACCCGTAC	799	AF008647

The reaction mixture (10 μL) contained 1×DreamTaq™ Green buffer, 1 u DreamTaq™ polymerase (ThermoScientific), 200 μM deoxynucleoside triphosphates mix (dNTPs), 10 ng DNA sample, 0.2 μM each primer. The PCR was performed using T-Cy IQ5 (CreaCon, The Netherlands). The amplification parameters were: initial denaturation (96 °C) 2 min, 30 cycles: denaturation (94 °C) 45 s; annealing (55 °C) 30 s; elongation (72 °C) 1 min; final elongation (72 °C) 2 min. The products of the amplification reaction were visualized by electrophoresis in a 3% agarose gel in

0.5×TBE (tris-borate buffer solution). DNA electrophoresis has been carried out for 1.5 hour at an electric field intensity of 5 V cm^{-1} (Abramova, 2006; Prysiazhniuk et al., 2019). The size of amplicons was determined using TotalLab v2.01 software (trial version).

To test the non-specific PCR products which are obtained as a result of amplification with the studied markers, PCR in silico was carried out on maize mitochondrion DNA sequences of two fertile cytotypes NA and NB (GenBank: DQ490952.1 and AY506529.1) using the SnapGene software (trial version) (Kalendar et al., 2017).

RESULTS AND DISCUSSION

Field studies

Four pairs of sterile analogues of lines were obtained during four backcrossing RL106S (17M, 19M, 23M, 27M та 29M) and maintainers RL106fS (18M, 20M, 24M, 28M and 30M) for S type of CMS and RL108C (195C, 201C, 205C, 207C, 209C) and RL108fC (196C, 202C, 206C, 208C, 210C) for C type of CMS respectively. Sterility control of groups of each backcrossing cycle was conducted in a breeding nursery to exclude cases of late emergence of fertile anthers on lateral branches during the drying of silk on ears.

The evaluation of the sterility maintain was carried out on hybrid combinations, which had as a parent component a sterile inbred line of maize, and as pollinators, inbred maintainer of line of the corresponding type. For S type, the following combinations were obtained: RL23S×RL106fS, RL107S×RL106fS, RL98S×RL106fS, RL105S×RL106fS, RL113S×RL106fS and for C type: RL109C×RL108fC, RL110C×RL108fC, RL112C×RL108fC, RL114C×RL108fC, RL115C×RL108fC. The obtained hybrid combinations were planted the following year in a control nursery for field assessment. According to the results of the field assessment, all the obtained hybrids were sterile.

The possibility of fertility restoration was studied on hybrid combinations RL23S×RL106fS and RL105S×RL106fS by pollination of S type inbred restorer of line RL34SB and RL109C×RL108fC and RL110C×RL108fC – by C type inbred restorer of line RL77CB. The used lines which were growing in the control nursery were identified as fertile.

Laboratory studies

As a result of the analysis of maize lines, which showed sterility in the field, amplicons of the expected size 799 bp were obtained using primers to S type of sterility (Fig. 1 and Fig. 2).

As shown in Fig. 1, maize lines that have a sterile cytoplasm of S type showed the presence of 799 bp DNA fragment. Amplicon 799 bp was identified in sterile analogues of lines: 17M, 19M, 23M, 27M and 29M. In fertile lines which are maintainers, from 2 to 3 amplicons have been identified. DNA fragments of size 632, 855, and 1,088 bp were identified in lines 18zM, 28zM, and 30zM; two amplicons, 855 and 1,145 bp were detected in lines 20zM.

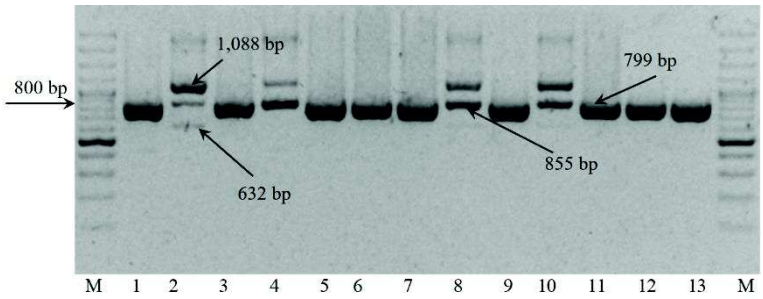

Figure 1. Results of PCR of maize lines with S type of sterility: M – 100 bp DNA Ladder O'GeneRuler (Thermo Scientific); 1, 3, 5, 6, 7, 8 – sterile analogues of lines of S type; 2, 4, 8, 10 – fertile lines of S type; 11, 12, 13 – sterile lines of S type.

In all sterile lines with S type of cytoplasm (RL23S, RL107S, RL98S, RL105S and RL113S), amplicons of the expected size 799 bp were identified (Fig. 2). Sterility based single-cross hybrids and restored hybrids of S type have been shown the presence of typical amplicon 799 bp as well.

It was noted that the 24zM line, which was selected as fertile in the field, turned out to be sterile by PCR with 799 bp amplicons. According to the classification of maize fertility levels according to Gontarovsky (1971), lines are completely sterile when all or almost all of the anthers are completely or partially in spikelets (0 and 1 class). There is a class 'incomplete fertility', in this case the number of fertile anthers does not exceed 25%. It is also known that environmental factors significantly affect the expression of sterility in S type lines. Whereas, the 24zM line was determined to be fertile in the field, but showed a sterile cytoplasm of S type by PCR.

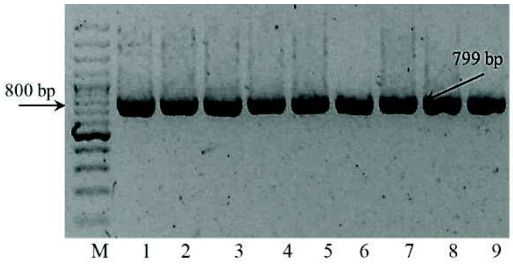

Figure 2. Results of PCR of maize sample with S type of sterility: M – 100 bp DNA Ladder O'GeneRuler (Thermo Scientific); 1, 2 – sterile lines of S type; 3–7 – sterility based single-cross hybrids (S type); 8–9 – restored hybrids (S type).

According to the results of PCR analysis of lines and hybrids with C type of sterility, the obtained amplicons were 398 bp (Fig. 3 and Fig. 4).

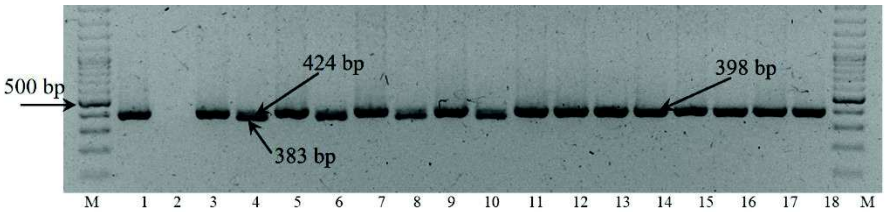

Figure 3. Results of PCR of maize sample with C type of sterility: M – 100 bp DNA Ladder O'GeneRuler (Thermo Scientific); 1, 3, 5, 6, 7, 8 – sterile analogues of lines of C type; 2, 4, 8 – fertile lines of C type; 11–15 – sterile lines of C type; 16–18 – sterility based single-cross hybrids (C type).

According to obtained data, in all sterile analogues of lines of C type (195C, 201C, 205c, 207c, 209C), an amplicon of expected size 398 bp was detected. In fertile lines 202zC, 206zC, 208zC and 210zC, two amplicons of sizes 383 and 424 bp were identified. In the fertile line 196 zC, in contrast to others, by markers to C type of sterility, any amplification product was not found. The presence of amplicon 398 bp was also identified in sterile lines with C type of sterility (RL109C, RL110C, RL112C, RL114C and RL115C). In sterility based single-cross hybrids of C type (RL109C× RL108fC, RL110C×RL108fC and RL112C×RL108fC), the amplicon of size 398 bp was detected (Fig. 3 and Fig. 4).

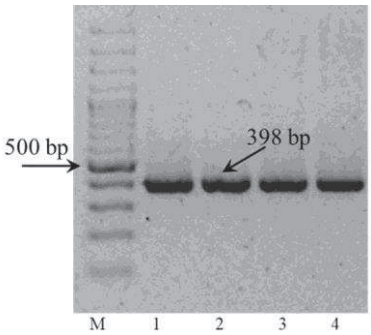

Figure 4. Results of PCR of maize sample with C type of sterility: M – 100 bp DNA Ladder O'GeneRuler (Thermo Scientific); 1–2 –sterility based single-cross hybrids (C type); 3–4 – restored hybrids (C type).

In Fig. 4, the presence of a amplicons of size 398 bp in sterility based single-cross hybrids RL114C×RL108fC and RL115C×RL108fC, as well as in restored hybrids (RL109C×RL108fC)× RL77CB та (RL110C×RL108fC)× RL77CB are shown. Therefore, the presence of amplicons of the expected size in sterility based single-cross hybrids and restored hybrids indicates the possibility of using DNA markers to evaluate hybrids on the method of breeding and determination of the type of sterility of lines (S and C), which formed the basis of a particular hybrid.

According to results of the studies, it was found that in the maintainers of lines of C type of sterility with relevant primers, two amplicons were identified which had the same size for all samples. Similar results were also obtained with primers to S type of sterility, however, the sizes of amplicons in that type of sterility in the maintainers of lines vary. According to the results obtained by Liu et al. (2002), in fertile lines, no amplicons were found by markers to S and C types of sterility. Also, the absence of any amplification products in fertile lines was noted by Slischuk et al. (2011). They were investigating 88 lines of maize, which included fertile lines, sterile analogues of lines, maintainers and fertility restorers of lines of Ukrainian and USA breeding. In our study, no amplicon was identified in one fertile line only with a marker to C type of sterility.

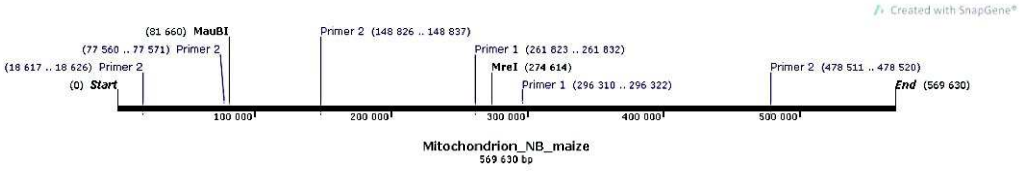

Figure 5. Results of PCR in silico of mitochondrion DNA sequence to NB cytotype with primers to C type of sterility.

To test the hypothesis whether there is a certain pattern concerning identified amplicons in fertile lines by markers to C and S types of sterility, PCR in silico was carried out on maize mitochondrion DNA sequences of two fertile cytotypes NA and

NB. Figs 5 and 6 show the results of PCR in silico with primers to cytotype NB as more common type among commercial lines and hybrids (Clifton et al., 2004).

According to obtained data, 2 to 4 binding sites were detected by testing primers to C type of sterility on the mitochondrion DNA sequence of NB cytotype. From 2 to 12 binding sites were determined by primer to S types of sterility (Fig. 6).

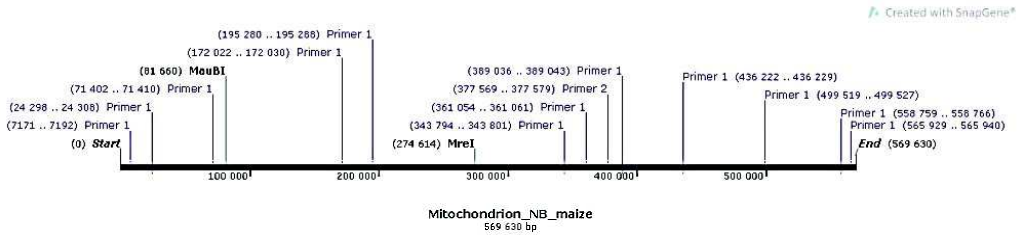

Figure 6. Results of PCR in silico of mitochondrion DNA sequence to NB cytotype with primers to S type of sterility.

It should be noted that an amplification product of size less than 50 kb (11,500 bp) was obtained only as a result of PCR in silico with primers to S type of sterility. According to the results of PCR in silico of mitochondrion DNA sequence to NA cytotype with primers to C and S types of sterility, from 2 to 4 binding sites were obtained and no amplification products of size less than 50 kb were detected.

Vancetovic et al. (2013) studied maize lines with S type of sterility in order to search for new sources of sterility. As a result of analysis of 24 different sources, additional amplicons were identified by authors, which differ from size of typical amplicons of the S type of sterility (799 bp). The authors suggested that the obtained profile is a consequence of the duplication of a smaller part of the mitochondrial genome of S type of CMS (Dewey et al., 1991; Darracq et al., 2010; Vancetovic et al., 2013).

Thus, as described above, there are about 20 sources of S type of sterility (Levings & Sederoff, 1983; Vancetovic et al., 2013) and Beckett (1971) also classified two types of C type of sterility, it can be assumed that the presence of nonspecific amplification products in fertile lines is explained by the presence of certain mitochondrion DNA sequences which are associated with various sources of sterility, which may have been included in the breeding process of the studied lines (Beckett, 1971; Krivosheev, 2018). Therefore, the presence of amplification products of any size which differs from products indicated the type of sterility is uninformative and cannot be used as a marker characteristic.

CONCLUSIONS

As a result of the studies, it was determined that DNA markers to C and S types of sterility are a quick and reliable approach for identifying sterile maize lines, in contrast to field studies, which have a number of limitations (temperature, humidity, sowing dates, daylight hours). Furthermore, it was found that the studied markers are equally efficient for the analysis of sterility based single-cross hybrids and restored hybrids. The use of DNA markers for determination of sterility is especially useful as part of a qualification examination of DUS. This approach makes it possible to quickly determine

the difference between lines which are morphologically identical and differ only by the type of cytoplasm, and also to determine the type of CMS based hybrid.

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