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Research Article

Molecular classification of Maize cytoplasms in a breeding program

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Abstract
Cytoplasmic male sterility (CMS) is maternally inherited in most of higher plants species. Together with nuclear restorer genes (*Rf*), CMS cytoplasms contribute significantly to the efficient production of hybrid seed. Three main types of male sterile cytoplasms are known in maize: T, S and C, which can be distinguished by crossing with specific restorer lines. Recently, PCR markers have been developed allowing the identification of different cytoplasms quickly and accurately. Our objective was to classify the cytoplasm type of maize inbred lines used in our breeding program and F1s obtained from crosses between CMS lines and elite maize lines using PCR multiplex. A multiplex PCR protocol was optimized for our conditions. We obtained the molecular classification of the analyzed cytoplasms. The optimized protocol is a valuable tool to trace male sterile cytoplasms and determine hybrid seed purity in our maize breeding program.

Keywords
Maize, cytoplasmic male sterility, molecular markers, hybrid seed production.

Introduction

Cytoplasmic male sterility (CMS) is a maternally inherited trait which determines the breakdown of functional pollen production (Rhoades, 1933). CMS is a good example of the interactions between nuclear and mitochondrial genome in plants. While male sterility is caused by mitochondrial chimeric genes, fertility restoration depends on nuclear genes (*Rf* genes) which suppress the cytoplasmic dysfunction reducing the expression of CMS-associated genes. Moreover, CMS/Rf systems significantly increase the efficiency of hybrid seed production eliminating the need of emasculation and assuring normal seed set in F1 (Schnable and Wise, 1998). In maize, there are three main groups of male sterile cytoplasms: T (Texas), S (USDA) and C (Charrúa). These cytoplasms are defined according to which specific *Rf* genes cancel male sterility. CMS T is restored sporophytically by two complementary dominant restorers, *Rf1* and *Rf2*. CMS C is restored sporophytically by the dominant *Rf4* gene and by two additional independent genes, *Rf5* and *Rf6*. Restoration of CMS S is gametophytic and requires the dominant gene *Rf3*, though a novel thermo-sensitive restorer, *Rf9*, has been reported recently (Newton *et al.*, 2009; Gabay-Laughnan *et al.*, 2009). Traditional classification of male sterile cytoplasms by test crossing with lines carrying different *Rf* genes (Beckett, 1971) is time and labour consuming. Early attempts to improve the efficiency of cytoplasm discrimination used electrophoresis of mitochondrial DNA and mitochondrial proteins (Kemble *et al.*, 1980; Forde *et al*., 1980) as well as RFLPs (Borck and Walbot, 1982). However, these methods are not adequate for a breeding program due to their cost and time requirements. Recently, knowledge of the molecular basis of CMS has allowed the design of specific primers corresponding to the mitochondrial DNA regions associated with each type of male sterile cytoplasm -the T-*urf13* region in T cytoplasm (Dewey *et al.* 1986**)**, the 5´ *atp6* C region in C cytoplasm (Dewey *et al.* 1991**)** and the *orf 355* region in S cytoplasm **(**Zabala *et al.* 1997**)**, thus making possible a quick and confident identification of different cytoplasms through a single PCR multiplex reaction (Liu *et al*., 2002).

Our maize breeding program has begun to develop CMS/*Rf* systems to increase the efficiency of hybrid seed production. In this context, we are currently incorporating CMS-C and CMS-S cytoplasms and *Rf* genes in our elite lines by backcross. We have introduced 5 inbred lines carrying male sterile cytoplasms C and S as well as the corresponding maintainer lines carrying normal cytoplasm to maintain them. Crosses were made to obtain the F1s between CMS-C and CMS-S genotypes and a group of elite lines We have also introduced 13 inbred lines carrying *Rf* genes on different CMS cytoplasms to use them as donors of restorer genes., The objective of this work was to classify the cytoplasm type of maize inbred lines used in our breeding program and to trace these cytoplasms in F_1 seed using PCR multiplex.

Material and Methods

Plant material: Five maize inbred lines carrying CMS-S and CMS-C as well as their maintainer lines and thirteen inbred lines carrying *Rf* genes on CMS-S and CMS-C cytoplasms were obtained from the Maize Genetics and Genomics Database - Maize GDB- (Lawrence *et al.,* 2007). Maize elite lines were obtained from breeding programs at INTA. Eleven F1s between CMS donor lines and elite lines were obtained at the experimental field (Table 1).

Molecular analysis: Total DNA was extracted from leaves of seedlings according to Saghai-Maroof *et al.,* (1984). The following primers developed by Liu *et al,* (2002) were used in a single multiplex reaction:

CMSTF5´CATGAAATGGGTGAAGTCTCTTTC-3´
CMSTR5´AAGAGAAAGGGAGACTTTGGTCCC-3´
CMSCF 5´-ATGCTAATGGTGTTCCGATTCC-3´
CMSCR 5´-AGCATCATCCACATTCGCTAG-3´
CMSCR 5´-AGCATCATCCACATTCGCTAG-3´
CMSSR 5´-AGTTCGTCCCATATACCCGTAC-3´

The expected amplicons for cytoplasms T, C and S were 440 pb, 398 pb and 799 pb, respectively (Liu *et al*., 2002). The PCR mix was as in Liu *et al,* (2002) except that no additives were added to enhance the specificity of the reaction. T*aq* DNA polymerase was from Fermentas, Vilnius, Lithuania. The thermal profile was as follows: 94ºC for 3 min followed by 35 cycles at 94ºC for 1 min, 55ºC for 30 sec, 72ºC for 1 min, with a final extension at 72ºC for 10 min. PCR products were separated on 1.5% (v/w) agarose gels stained with ethidium bromide and visualized under UV light. A pair of universal primers amplifying a region of mitochondrial DNA *(nad4* exon 1: 3´-CAGTGGGTTGGTCTGGTATG-5´; *nad4* exon 2: 3´-TCATATGGGCTACTGAGGAG-5´) was used following Demesure *et al*., (1995). All reactions were carried out in triplicate using independent DNAs.

Results and Discussion

Identification of male sterile cytoplasm type by test crossing is time consuming, labour intensive and not precise. Development of molecular methods based on PCR amplification of specific mitochondrial DNA regions associated with each type of male sterile cytoplasm allow a more effective classification of CMS types. PCR markers for cytoplasm type discrimination have been developed in onion (Havey, 1995; Engelke *et al*., 2003), pepper (Kim and Kim, 2005), rye (Stojalowsky *et al*., 2002) and rice (Dwivedi *et al*., 2008). In maize, Liu *et al*., (2002) developed a PCR multiplex assay for the determination of cytoplasm types and applied it to a wide range of inbred lines. In order to identify the cytoplasm types in our set of inbred lines we optimized the protocol by Liu *et al.,* (2002), shortening the time of the PCR reaction, using both reduced cycling times and fewer cycles. As expected, ~ 400 and 800 bp products were observed in lines carrying C and S cytoplasm respectively, whereas no product was obtained in lines with normal cytoplasm (Figure 1).

As no product is amplified from the normal type cytoplasm with this assay, we amplified another mitochondrial sequence common to all cytoplasm types, corresponding to an intron located between the exons 1 and 2 of the gene *nad4* (Demesure *et al*., 1995). Amplification of this region produced the expected ~ 1.7 kb product in all inbred lines (Figure 2), thus providing a PCR control for lines with normal cytoplasm. Our results confirmed previous data about cytoplasm type provided by MaizeGDB and by maize breeding programs at INTA (Table 1). Eleven crosses between CMS inbred lines and elite lines were made and F1 seedlings were used to check their cytoplasm types. As expected for a maternally inherited trait, the hybrids had the same cytoplasmic type as their mother plant (Figure 3). Our results corroborated the usefulness of this technique to trace sterile cytoplasms during their introgression into elite lines and to determine genetic purity of F1 seed. Ensuring the genetic purity of parental lines and hybrids is a prerequisite to realizing the full potential of the hybrids. Several PCR-based markers have been reported in rice which not only discriminate between CMS lines and their cognate maintainer lines, but also serve to assess the genetic purity of the hybrids (Dwivedi *et al.,* 2008).

Figure 4 shows the cytoplasm type of 13 inbred lines carrying *Rf* genes on different CMS cytoplasms. Molecular classification of the cytoplasm type agreed with data provided by MaizeGDB and by maize breeding programs at INTA (Table 1). These lines will be chosen as donors of *Rf* genes based on their efficiency of fertility restoration in our field conditions. Moreover, they will be used to develop segregating populations in order to look for molecular markers closely associated to *Rf* genes. These markers will be used in the future to apply marker-assisted selection during the development of restorer lines, in order to enhance the efficiency of hybrid seed production.

Besides their value to increase the efficiency of hybrid seed production, mitochondrial DNA markers are also useful to determine cytoplasm diversity amongst germplasm accessions and to identify new sources of cytoplasms inducing male sterility (Xu *et al*., 1995). Recently, Vancetovic *et al*., (2010) used this assay to characterize over 100 sources of CMS in a maize gene bank and found that predominant cytoplasmic male sterility type among the analyzed accessions was CMS-S. These authors stated that the PCR approach enabled a simple, fast and reliable large scale screening of

maize cytoplasm, significantly reducing time for cytoplasm characterizations compared to classical method of testing with restorers for each known type of CMS.

In conclusion, we have optimized a multiplex PCR protocol for our conditions which allowed us to classify not only the cytoplasm types of inbred lines of maize but also those of F1s plants. The optimized protocol is a valuable tool both to screen maize cytoplasms and to asses hybrid seed purity in our maize breeding program.

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Table 1. List of cultivars evaluated. Identification includes the cytoplasm type from the seed stock records.

No	Stock	Identification
1	CX36B	N6-cms-S *rf1 Rf2 rf3 RfC*
2	CX36D	B37-cms-S *rf1 Rf2 rf3 rfC*
3	CX36E	Wf9-cms-C *rf1 rf2 rf3 rfC*
4	CX36C	B37-cms-C *rf1 Rf2 rf3 rfC*
5	C836G	Mo17-cms-C *rf1 Rf2 rf3 rfC*
6	C836B	Wf9-mito-N *rf1 rf2 rf3 rfC*
7	C836F	Mo17-mito-N *rf1 Rf2 rf3 rfC*
8	C936F	N6-mito-N *rf1 Rf2 rf3 RfC*
9	C736C	B37-mito-N *rf1 Rf2 rf3 rfC*
10	LP 236	Elite line Pergamino-Mito-N
11	LP 2542	Elite line Pergamino-Mito-N
12	LP 122-2	Elite line Pergamino-Mito-N
13	LP 918	Elite line Pergamino-Mito-N
14	LP 917	Elite line Pergamino-Mito-N
15	LP 562	Elite line Pergamino-Mito-N
16	LP 1936	Elite line Pergamino-Mito-N
17	LE 08-307	Elite line Castelar-Mito-N
18	LE 08-306	Elite line Castelar-Mito-N
19	5 x 16	F1 (Mo17-cms-C x LP 1936)
20	5 x 12	F1 (Mo17-cms-C x LP 122-2)
21	5 x 15	F1 (Mo17-cms-C x LP 562)
22	5 x 13	F1 (Mo17-cms-C x LP 918)
23	3 x 15	F1 (Wf9-cms-C x LP 562)
24	3 x 10	F1 (Wf9-cms-C x LP 236)
25	4 x 14	F1 (B37-cms-C x LP 917)
26	4 x 15	F1 (B37-cms-C x LP 562)
27	4 x 11	F1 (B37-cms-C x LP 2542)
28	2 x 18	F1 (B37-cms-S x LE 08-306)
29	1 x 17	F1 (N6-cms-S x LE 08-307)
30	C736EA	Tr-CMS-S/ *rf1 rf3 Rf3 rfC*
31	C437F	A632-CMS-S *Rf3 rfC*
32	C437I	A634-CMS-S *Rf3 rfC*
33	C437O	H95-CMS-S *Rf3 RfC*
34	C437U	Ky21-CMS-S *Rf3 RfC*
35	C537L	Oh45-CMS-S *Rf3 RfC*
36	C537V	Va58-CMS-S *Rf3 rfC*
37	CX36A	N6 CMS-C *rf1 Rf2 rf3 RfC*
38	C437N	H95 CMS-C *Rf3 RFC*
39	C437P	K55 CMS-C *rf3 RfC*
40	C537B	M14 CMS-C *rf3 RfC*
41	C537K	Oh45 CMS-C *Rf3 RfC*
42	C637D	W64A CMS-C *rf3 RfC*

Figure 1. Identification of cytoplasm types in maize inbred lines by PCR. Lanes 1 to 18 correspond to inbred lines mentioned in Table 1. Lane 19: positive control includes DNA from inbred lines 1, 3 and 8. L: 100 bp ladder.

Figure 2. Amplification of a *nad 4* region in maize inbred lines. Lanes 1 to 18 correspond to inbred lines mentioned in Table 1. L: 1 kb ladder.

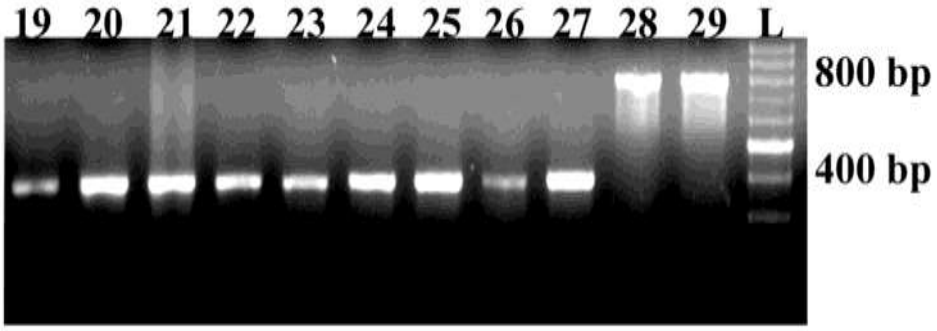

Figure 3. Identification of cytoplasm types in F1s by PCR. Lanes 19 to 29 correspond to hybrids mentioned in Table 1. L: 100 bp ladder.

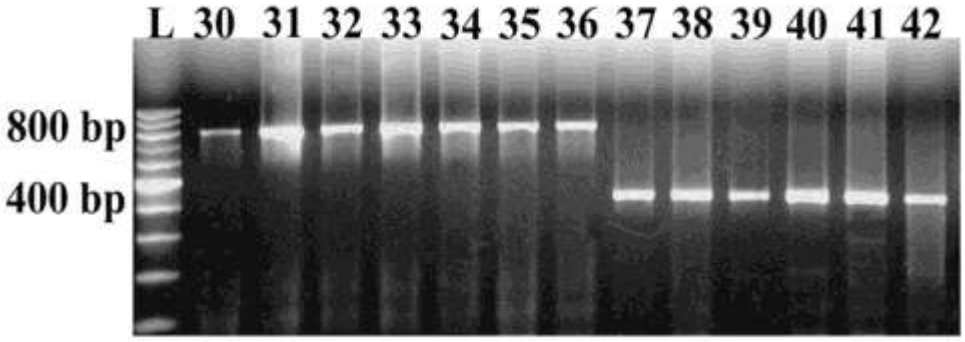

Figure 4. Identification of cytoplasm types in maize inbred lines by PCR. Lanes 30 to 42 correspond to inbred lines mentioned in Table 1. L: 100 bp ladder.