

A PCR Assay for Rapid Discrimination of Sterile Cytoplasm Types in Maize

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ABSTRACT

A single-seed, multiplex polymerase chain reaction (PCR) protocol is presented to distinguish the three major types of cytoplasmic male sterility (CMS-T, -C, and -S) in maize (*Zea mays* L.). Three pairs of PCR primers were designed, corresponding to the chimeric regions of mitochondrial DNA sequences specific for the three types of CMS cytoplasm. The specificity of these primers was tested by means of the inbred line B37 with male-sterile (T, C, and S) and normal (N) cytoplasm as reference material. Subsequently, a single-seed PCR assay was developed for the determination of cytoplasm types in a single reaction and applied to a wide range of inbred lines with normal and sterile cytoplasm (T, C, and S) and with predominantly identified subtypes of the major sterile cytoplasm types. Our protocol proved to be a rapid, inexpensive, and reliable procedure for the molecular classification of male sterile maize cytoplasm to the three major categories T, C, and S.

CYTOPLASMIC MALE STERILITY has been identified and characterized in many crop species and has been used successfully for the commercial production of hybrid seed (Kaul, 1988). Maize has three major types of male-sterile cytoplasm: CMS-T (Texas), CMS-S (USDA) (Duvick, 1965), and CMS-C (Charrua) (Beckett, 1971). CMS-T was widely used in the maize seed industry prior to 1970s but its use has been greatly reduced because it confers susceptibility to *Bipolaris maydis* (Nisikado & Miyake) Shoemaker (= *Helminthosporium maydis* Nisikado & Miyake) (Ullstrup, 1972). As a consequence, mainly C- and S-type cytoplasm is used as sterility source for the production of maize hybrid seed today. Therefore, a procedure to determine reliably the type of cytoplasm in the breeding material could be useful.

Tester lines containing nuclear Rf (restorer of fertility) genes are traditionally used to determine and classify the CMS types (Beckett, 1971) and still, test crossing is the most conclusive method for categorizing maize cytoplasm. However, the test-crossing procedure is time consuming and labor intensive. Knowledge about the molecular structures and the mechanisms underlying CMS increased considerably with the development of molecular approaches. Mutations responsible for CMS have been shown to reside in the mitochondrial DNA (mtDNA) in many plant species (Schnable and Wise, 1998). Unique characteristics of the mitochondria, particularly the mitochondrial genome structure and gene expression, were identified and used to distinguish the major types of maize cytoplasm (Levings and Prings, 1976; Forde et al., 1978). The chimeric gene *T-urf13* was detected in mtDNA of CMS-T (Dewey et al. 1986) and an open reading frame (ORF) of unknown origin was

found in a chimeric *atp6 / atp9* region of CMS-C (Dewey et al., 1991). A repeated mtDNA region 'R', containing two chimeric ORFs, i.e., ORF355 and ORF77, was found in CMS-S (Zabala et al., 1997). Within each CMS type, but particularly in CMS-S, various subtypes have been defined by variations in the degree of fertility of the F₁ progenies from crosses with different tester lines carrying restorer genes (Beckett, 1971; Sisco et al., 1985). Partially, these subtypes could be further characterized by molecular means (Sisco et al., 1985).

The polymorphism in mtDNA can be assessed much more rapidly by molecular markers, e.g., RFLP (restriction fragment length polymorphism) or PCR-based markers, than by the traditional test-crossing procedure. Unfortunately, RFLP analysis is too time consuming to be applied routinely in breeding programs. Recently, PCR-based markers have been suggested to be a rapid and definitive identification of the cytoplasm of individual plants (Nakajima et al., 1999; Sato, 1998; Alcalá et al., 1997). To our knowledge, no work has been done on the detection of the major types of maize cytoplasm by PCR. On the basis of the available chimeric mtDNA sequences specific for the various male-sterile cytoplasm, a single-seed multiplex PCR approach was developed for a rapid and reliable identification of CMS types in maize breeding programs.

MATERIALS AND METHODS

Plant Material

CMS and fertile maize inbred lines with known types of cytoplasm (N, T, C, and S) were obtained from seed stock centers in the USA and France (Table 1). Furthermore, 73 fully male-sterile inbred lines were obtained from the Cornell Cyto-Stock collection maintained at the Maize Genetics Cooperation Stock Center, University of Illinois, Urbana, IL (Table 2). The cytoplasm of most of these lines belonged to previously described subtypes of T, C, or S (Beckett, 1971; Sisco et al., 1985).

Primer Design

Three sets of PCR primers specific for T-, C-, and S-type cytoplasm were designed according to the published mtDNA sequences in the GenBank (M12582, S81074, AF008647) and synthesized by MWG-Biotech AG, Germany, and are as follows.

CMSTF	5'-CATGAAATGGGTGAAGTCTCTTTTC-3'
CMSTR	5'-AAGAGAAAGGGAGACTTTGGTCCC-3'
CMSCF	5'-ATGCTAATGGTGTCCGATTCC-3'
CMSCR	5'-AGCATCATCCACATTCGCTAG-3'
CMSSF	5'-CAACTTATTACGAGGCTGATGC-3'
CMSSR	5'-AGTTCGTCCCATATACCCGTAC-3'

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Abbreviations: CMS, cytoplasmic male sterility; mtDNA, mitochondrial DNA; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

The binding sites of these primers on the mtDNA are indicated in Fig. 1.

The structural differences between the mtDNAs 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.

DNA Isolation

Mitochondrial DNA was extracted from etiolated shoots according to Dewey et al. (1986). Total DNA was extracted from leaf and coleoptiles of seedlings by the cetyltrimethylammonium bromide (CTAB) method (Saghai-Marooof et al., 1984). Seed DNA was isolated from a whole seed or the distal half of a seed. The seed material was crushed into pieces and extracted with 500 mM Tris-HCl (pH 7.5), 350 mM NaCl, and 0.5% (w/v) SDS in a 1.5-mL Eppendorf tube. After adding potassium acetate to a final concentration of 0.5 M and incubating on ice for 20 min, seed DNA was precipitated with ethanol after chloroform:isoamylalcohol (24:1) extraction and diluted approximately 1:100 with H₂O for the PCR reaction.

Polymerase Chain Reaction and Electrophoresis

PCR reactions were performed in 25- μ L volumes containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 μ M MgCl₂, 200 μ M of each deoxynucleotide, 50 ng of each primer (totally 6 primers), about 50 ng template DNA, and 1 U Taq DNA polymerase. To enhance the specificity of the reaction the PCR additive, 'TaqMaster' (Brinkmann Instruments BioSystems Division, Westbury, NY) was routinely added according to the recommendation of the supplier. The amplification was performed in an Eppendorf Mastercycler using the following program: 94°C for 2 min followed by 40 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min and 50 s, with a final extension at 72°C for 5 min. PCR products were separated on 1.4% (w/v) agarose gels and visualized under UV light after ethidium bromide staining.

Table 1. PCR identification of male sterile cytoplasm types of inbred lines with known cytoplasm and genetic background. When no PCR product was obtained, the material was classified as male fertile N cytoplasm.

Genotype	Sterile/ Fertile [†]	Cytoplasm (recorded)	Cytoplasm (PCR)	Source [‡]
A188	Fertile	N (=NA)	N	a
B37	Fertile	N (=NB)	N	a
B37	Sterile	T	T	a
B37	Restored	T	T	a
B37	Sterile	C	C	a
B37	Sterile	S	S	a
N6	Fertile	N	N	a
N6	Sterile	T	T	a
N6	Restored	T	T	a
N6	Restored	C	C	a
N6	Sterile	S	S	a
Mo17	Fertile	N	N	a
Mo17	Sterile	C	C	a
Mo17-RB	Sterile	RB [§]	C	a
Mo17	Sterile	S	S	a
Wf9	Fertile	N	N	a
Wf9	Sterile	T	T	a
Wf9	Restored	T	T	a
Wf9	Sterile	S	S	a
SK2	Fertile	N	N	a
SK2	Sterile	T	T	a
SK2	Restored	T	T	a
Tr	Fertile	N	N	a
Tr	Restored	S	S	a
F2	Fertile	N	N	b
F2	Sterile	C	C	b
F7	Fertile	N	N	b
F7	Sterile	T	T	b

[†] Fertile lines containing sterile cytoplasm are restored to fertility by nuclear restorer genes Rf1 + Rf2, Rf3, and RfC in the cases of cytoplasm T, S, and C, respectively.

[‡] (a) Maize Genetic COOP Stock Center, USA; (b) INRA, France.

[§] The provider of the material reported RB to be of C-type.

RESULTS

Evaluation of the PCR Primers

Three sets of PCR primers specific for mtDNA of maize CMS-T (CMSTF + CMSTR), CMS-C (CMSCF +

Table 2. A PCR-based classification of male sterile cytoplasm types in sterile inbred lines obtained from the Cornell Cyto-Stock collection. Cornell code is the code of the nuclear genotype plus the recorded cytoplasm type.

Cytoplasm (recorded)	Lines (Cornell code)	Cytoplasm (PCR)
T	A619-T, CO150-T, W64A-T	T
HA	A619-HA, A632-HA, B14AHt-HA, B37Ht-HA, CO220-HA, MS64-7-HA, W64A-HA	T
ij2	A619-ij2, A632-ij2, B14AHt-ij2	T
Q	A619-Q, A632-Q, B37Ht-Q, W64A-Q	T
RS	A619-RS, CO150-RS, CO220-RS, MS64-7-RS, W64A-RS	T
C	B14AHt-C, B37Ht-C, MS64-7-C, NYD410-C	C
Bb	CO220-Bb	C
CR	A632-CR, B14AHt-CR	C
PR	CO220-PR	C
RB	B37Ht-RB, MS64-7-RB, NYD410-RB	C
RPS	NYD410-RPS, W64A-RPS	C
S	B37Ht-S, W64A-S	S
CA	B37Ht-CA, NYD410-CA, W64A-CA	S
D	NYD410-D, W64A-D	S
EK	B37Ht-EK, W64A-EK	S
G	B37Ht-G, NYD410-G, W64A-G	S
H	CO150-H, NYD410-H, W64A-H	S
I	NYD410-I	S
IA	NYD410-IA, W64A-IA	S
J	B37Ht-J	S
K	B37Ht-K, NYD410-K	S
M	W64A-M	S
ME	B37Ht-ME, NYD410-ME, W64A-ME	S
ML	B37Ht-ML, NYD410-ML	S
MY	B37Ht-MY, W64A-MY	S
PS	B37Ht-PS, Co150-PS, NYD410-PS, W64A-PS	S
SD	B37Ht-SD, NYD410-SD, W64A-SD	S
TA	W64A-TA	S
TC	W64A-TC	S

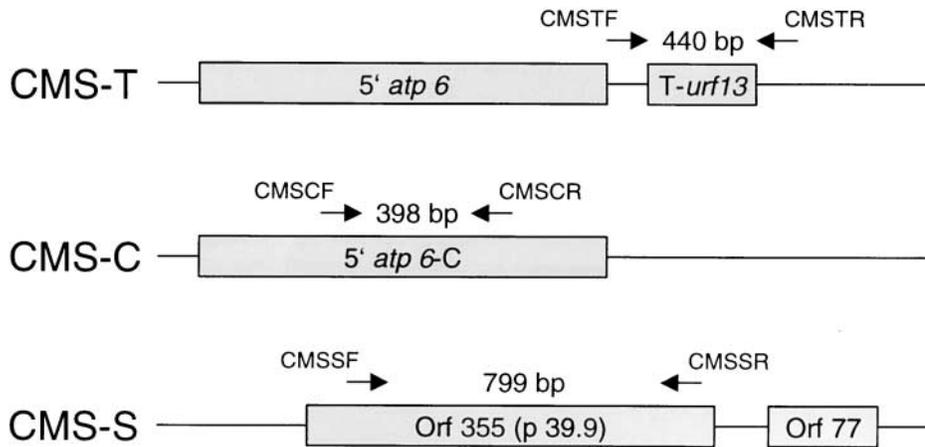


Fig. 1. Recombinant mtDNA regions of the sterile cytoplasm types T, C, and S. Black arrows indicate the binding sites of the specific primer pairs; the sizes of the expected amplification products are given between the arrows.

CMS-C (CMSCF + CMSCR) and CMS-S (CMSSF + CMSSR) were evaluated separately with isolated mtDNA, total leaf DNA, and total seed DNA of the inbred line B37 with four different types of cytoplasm (N, T, C, and S). No primer pairs amplified fragments from the DNAs of mitochondria, total leaves, and seeds of B37 with N-type cytoplasm. As designed, the primer set CMSTF and CMSTR yielded only a 440-bp DNA fragment in B37 with T-type cytoplasm, while the primer sets CMSCF and CMSCR, CMSSF, and CMSSR yielded unique 398- and 799-bp DNA fragments in B37 with C- and S-type cytoplasm, respectively (Fig. 2). No differences in the PCR amplification was observed when either mtDNA, total leaf DNA, or seed DNA was used as template.

Single-Seed Multiplex PCR

A multiplex PCR assay combining all three sets of primers in a single reaction was applied to total seed DNA from a large set of inbred lines with known cytoplasm and genetic background. All four different types of cytoplasm could be clearly identified. No DNA frag-

ment was amplified in lines with N-type cytoplasm, regardless of whether the common NB type was used (e.g., from B37) or the less frequent NA type which is found in the inbred line A188 (Fauron et al., 1995). On the other hand, 398-, 440-, and 799-bp DNA fragments were detected in lines with C-, T-, and S-type cytoplasm, respectively. The PCR results were in full agreement with the histories of all tested lines from different sources (Table 1). Furthermore, three DNA fragments of the same sizes as those above were detected in one reaction when mixed DNA (T + C + S) was used as template (data not shown).

Classification of Sterile Cytoplasm Types

Following the single-seed multiplex PCR procedure, the cytoplasm of 73 sterile inbred lines from the Cornell Cyto-Stock collection (Table 2) were investigated. All recorded C-, S-, and T-type cytoplasm in the collection were confirmed by the PCR. All other cytoplasm, recorded with different nomenclature, could be categorized clearly into one of the three major male sterile cytoplasm types (Table 2).

DISCUSSION

Besides the traditional test-crossing procedure, biochemical and molecular protocols have been used for the discrimination of cytoplasm types in maize. These methods are still labor intensive, mainly because they require highly purified mitochondrial DNA. RFLP markers provide reliable means for the molecular characterization of N-, C-, T-, and S-type cytoplasm types in maize. The mtDNA probes specific for the corresponding cytoplasm can be used to distinguish each of the types of male-sterile cytoplasm in Southern hybridization. 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). However, RFLP analysis is expensive and time consuming and, for these reasons, not suitable for routine applications in breeding programs. On the other hand, PCR-based markers provide breeders with a quick and simple tool for screening and identifying unknown cytoplasm

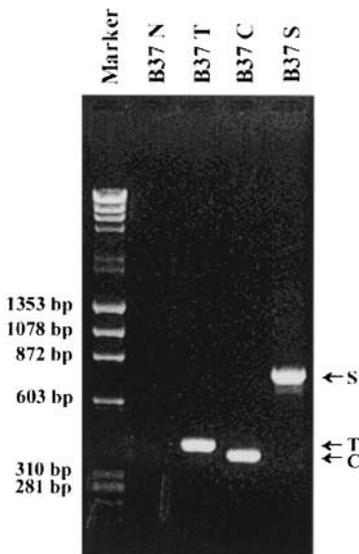


Fig. 2. Identification of the three sterile cytoplasm types in the inbred line B37 determined by PCR from crude seed DNA as template.

when mtDNA is used as the template (Nakajima et al., 1999; Sato, 1998). Single-seed PCR has been used to characterize the nuclear genome loci for marker assisted breeding (D'Ovidio, 1993; Wang et al., 1993; Sakamoto et al., 2000). To our knowledge, no report has been published on the characterization of mitochondrial genome sequences with single-seed PCR. We developed an approach using a combination of six primers in a single PCR 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, T, and S (Table 1). No DNA fragment is amplified from the N-type cytoplasm in our assay because a mtDNA fragment unique for the normal type does not exist. In case an internal control for N-type cytoplasm is required, we suggest to add an additional primer pair amplifying a conserved sequence common to all cytoplasm types. The pattern of amplified DNA fragments did not reveal any indication for genetic variability within the chimeric mtDNA regions of C-, T-, and S-type cytoplasm, since all tested lines matched the pattern of the respective B37 reference material (Table 1, Fig. 2). Our results on the Cornell Cyto-Stock material (Table 2) are in full agreement with the categorization of cytoplasm to major CMS types performed by Beckett (1971) and Sisco et al. (1985) on the basis of test crossing and RFLP analysis. Minor molecular differences between subtypes did not affect primer specificity or fragment length of PCR products.

The presented approach is fairly simple, rapid, reliable, and widely applicable for large-scale screening of maize cytoplasm in breeding programs and can also serve to test the purity of seed material.

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