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Heteroplasmy in the T-*urf13* mitochondrial gene of the Texas cytoplasm of maize

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ABSTRACT. In maize (*Zea mays* L.), the Texas (T) cytoplasm carries male sterility (cms-T) and susceptibility to host-specific fungal toxins and to the insecticide methomyl, traits of great interest both for basic research and plant breeding. These traits are apparently inseparable, and they are directly associated with the *T-urf13* mitochondrial gene, which encodes a 13-kilodalton protein (URF13). Reversion to male fertility, associated with toxin/methomyl resistance, has been observed in progenies of cms-T somaclones regenerated from methomyl resistant calli. Diversity for PCR amplification of *T-urf13* sequence and URF13 expression was observed within revertant progenies. Apparently, this diversity occurred among tissues within ear and tassel, and between ears and tassels of individual plants. The results demonstrate heteroplasmy in *T-urf13* sequence persisting through several generations of T-cytoplasm mutants, that apparently consisted of deletions and sequence alterations. Furthermore, URF13 deficiency suggested that total or partial cytoplasmic reversions to male fertility in cms-T somaclones are caused by T-*urf13* heteroplasmy.

Key words: heteroplasmy, maize, T-*urf13*, Texas cytoplasmic male sterility.

RESUMO. Heteroplasmia para o gene mitocondrial T-*urf13* do citoplasma texas de milho. Em milho (*Zea mays* L.), o citoplasma texas (T) condiciona macho-esterilidade (cms-T) e susceptibilidade à toxina de fungos e ao inseticida metomil, características de grande interesse para estudos básicos e melhoramento de plantas. Essas características são aparentemente inseparáveis e estão diretamente associadas ao gene mitocondrial *T-urf13*, que codifica uma proteína de 13 kilodaltons (URF13). Reversão para fertilidade masculina, associada à resistência à toxina/metomil, tem sido observada em progênies de somaclones cms-T, derivados de calos resistentes ao metomil. Foi encontrada diversidade para amplificação via PCR do gene *T-urf13* e para expressão da proteína URF13 nesses somaclones revertentes. Aparentemente, esta diversidade ocorre dentro de tecidos de espiga e panícula e entre espiga e panícula de uma mesma planta. Os resultados demonstram heteroplasmia na seqüência *T-urf13*, que persistiu em várias gerações desses mutantes, consistindo aparentemente de deleções e alterações na seqüência de DNA. Além disso, deficiência da proteína URF13 em somaclones férteis sugeriu que a reversão total ou parcial da macho-fertilidade é causada pela heteroplasmia no gene *T-urf13*.

Palavras-chave: heteroplasmia, milho, T-*urf13*, esterilidade masculina citoplasmática Texas.

Mitochondrial genomes of higher plants are larger, more variable in size, and more complex as compared to other organisms. Higher plant mitochondrial DNA (mtDNA) encodes a few respiratory polypeptides and ribosomal proteins that are encoded by nuclear genes in animals and fungi (Newton, 1988). Intramolecular and intergenomic recombinations involving repeats are the main forces generating diversity in plant mitochondrial genomes. Rare recombinational events coupled with selective amplification of preexisting molecules have been proposed as a cause of sudden genome reorganization, possibly leading to the evolution of cytoplasmic male sterility (cms) and other

mitochondrial variants (Levings III and Brown, 1989). Some of these mtDNA recombinations have resulted in a novel chimeric transcriptional unit in maize (*Zea mays* L.), called T-*urf13*, which is unique to plants carrying the Texas (T) cytoplasm (Dewey *et al*., 1986; Levings III, 1990). The coding region of the T-*urf13* gene consists of 88 codons homologous to an untranscribed 3'-flanking region of the 26 rRNA gene (*rrn26*), 9 codons of unknown region, and 18 codons with homology to the codon region of *rrn26*. Both T-*urf13* and *atp6* mitochondrial genes seem to have similar promoters, because their 5'-flanking region is almost identical in both genes (Dewey *et al*., 1986). In T-mitochondria, the T-*urf13* sequence is cotranscribed with another gene called *orf221*, which encodes a mitochondrial membrane protein apparently without association with T-cytoplasm traits (Dewey *et al.*, 1987; Prioli *et al*., 1993). The T-*urf13* sequence encodes a 13 kDa polypeptide (URF13) that is directly associated with the Texas cytoplasmic maize sterility (cms-T) of maize (Dewey *et al*., 1987; Wise *et al.*, 1987a). URF13 seems to be constitutively expressed in cms-T plants, because it has been found in all cms-T maize tissues analyzed.

For nearly two decades, from the 1950s to 1970, the Texas cytoplasmic male sterility (cms-T) of maize was extensively used, in combination with fertility restorer nuclear genes, in the production of commercial hybrid seeds. Because pollen is aborted in cms-T plants, though female fertility is not affected, this trait was used as a means of preventing hand and mechanical emasculation. The Texas cytoplasm provided a reliable and stable source of male sterility in the field. However, its use in agriculture was discontinued in 1970, after a disease known as southern corn leaf blight occurred in epidemic proportions specifically destroying maize carrying T-cytoplasm. This fungal disease is caused by *Bipolaris maydis* race T (formely known as *Helminthosporium maydis* race T). Another fungal pathogen, *Phyllosticta maydis*, is also virulent to cms-T. These pathogens produce toxins similar in structure, called HmT and Pm (T-toxins), respectively, which affect the membranes of T-mitochondria. The carbamate insecticide methomyl (S-methyl-N-[(methylcarbamoyl)-oxy]thioacetimidate] causes equivalent toxic effects in T-mitochondria, acting as a functional analog of T-toxin (for review see Laughnan and Gabay-Laughnan, 1983; Levings III, 1990).

It has been demonstrated in heterologous systems that the T-*urf13* mitochondrial gene is indeed responsible for the specific sensitivity to T-toxin and methomyl (Dewey *et al*., 1988; Huang *et al*., 1990; Korth *et al.*, 1991; Korth and Levings III, 1993; Chaumont *et al*., 1995). Evidence that *T-urf13* is associated with male sterility has come from demonstration that the *Rf1* nuclear gene, which specifically restores male fertility in plants carrying T-cytoplasm, alters the transcriptional profile of T-*urf13* and decreases the abundance of URF13 by approximately 80% (Dewey *et al*., 1987; Kennel *et al*., 1987). Strong evidence came also from cms-T revertant plants regenerated from tissue culture. The T-*urf13* sequence was deleted or mutated in these plants, which became male fertile and toxin/methomyl resistant (Gengenbach *et al.,* 1977; Umbeck and Gengenbach, 1983; Wise *et al*., 1987b; Fauron *et al*., 1990; Prioli *et al*., 1994). Although all evidences strongly correlate T-*urf13* with male sterility in cms-T plants, there are no conclusive experimental data indicating the mechanism by which the URF13 polypeptide causes pollen abortion (Levings III, 1990).

Cms-T is highly stable under field conditions; however in tissue culture it undergoes recombinational mutations (Wise *et al.*, 1987b; Fauron *et al.*, 1990; Prioli *et al.*, 1994) that can be useful to better understand the T-*urf13* effects in cms-T plants, as well as to study recombinational mechanisms in plant mtDNA. A possible consequence of mtDNA recombinational events in cms-T cells under tissue culture conditions is the occurrence of genetic variants that could persist in callus. These genetic variants could be transmitted to regenerated plants. The occurrence of two or more codominant types of molecules within the mitochondrial DNA population of the same tissue or of the same individual is called heteroplasmy. It has been described mostly in humans and animals (Asley *et al*., 1989; Comas *et al*., 1995; Grzybowski, 2000), and more rarely in plants (Marienfeld *et al.*, 1993; Gu *et al.*, 1995; Wintz, 1994). According to Fauron *et al*. (1990), reversion to fertility of a cms-T somaclone (V3) recovered from callus culture is due to a very precise rearrangement of its mitochondrial genome via recombination between two specific sets of repeats. The V3 revertant fertile somaclone was studied after several generations of self-pollination. As suggested by DNA analysis, these specific recombinational events could have eliminated the T-*urf13* sequence from this somaclone. Because no previous generations or sister lines were available or studied, it was not possible to find intermediate stages of recombination containing the heteroplasmic T-*urf13* sequences in these plants.

We have demonstrated that the T-*urf13* gene can

exist in heteroplasmic state in the T-cytoplasm of maize plants derived from methomyl resistant callus. Heteroplasmy for T-*urf13* gene could explain total and partial reversion to male fertility in cms-T somaclones derived from methomyl-resistant callus. The heteroplasmic state of T-*urf13* in these somaclones is of interest to study intermediate stages of recombination in mtDNA and the hypothesis of a selective elimination of T-*urf13* gene in heteroplasmic tissues.

Materials and methods

Plant materials. Cms-T maize revertant progenies of six somaclones and a progeny from a stable male-sterile somaclone were analyzed for heteroplasmy of T-*urf13* sequence. These progenies were derived from maize plants regenerated from callus of a hybrid genotype F_1(Cat100-1cms-T x T204). The Cat100-1cms-T parental inbred line was derived from Cateto maize race belonging to the Germplasm Bank of the Department of Genetics, State University of Campinas, SP, Brazil. The T204 is a Tuxpeño maize inbred line, which was gently provided by Dr. C. S. Levings III, Department of Genetics, North Carolina State University, USA. Occasionally, pollen of either the inbred line L922 or the hybrid F_1(Cat100-1 x L922) was used to pollinate somaclones. All maize lines used in this work were recessive for cms-T restorer nuclear genes. Donor plants were grown in the field.

Tissue culture and somaclone evaluation. Embryogenic callus cultures were initiated from immature embryos. The culture medium (CM-1) consisted of N6 salts (Chu *et al.*, 1975), glycine (30 μM), thiamine-HCl (15 μM), nicotinic acid (7.5 μM), pyridoxine (7.5 μM), inositol (550 μM), 2,4-D (dichloro-phenoxyacetic acid) (10 μM), sucrose (30 g/L), and gelrite (2.3 g/L). The pH was adjusted to 5.8 before autoclaving. Excised embryos were placed scutellar side upwards onto about 25 mL of medium in plastic Petri dishes (100 mm x 20 mm). Cultures were incubated in the dark, at 28 $\pm$1 ºC, and subcultured every 15 to 20 days. Selective medium consisted of CM-1 medium containing 0.65 mM or 0.9 mM methomyl. Cultures were incubated on selective medium, under 14/10 h light/dark photoperiod (approx. 125 $\mu E.m^{-2}.s^{-1}$), at 28 $\pm$1 ºC. After 20 to 25 days, callus sectors growing on selective medium were transferred to fresh selective culture medium. Resistant callus sectors were kept for a few weeks on selective medium, or transferred to regeneration medium if somatic embryos had started to germinate. Regeneration medium (RM) consisted of MS (Murashige and Skoog, 1962) salts and vitamins, inositol (550 μM), sucrose (20 g/L), and gelrite (2.3 g/L). Cultures on RM medium, contained in 350 mL jars, were incubated under light conditions. Regenerated plants (R_0) were transferred to greenhouse or to the field. Fertile plants were self-pollinated whenever possible. Male sterile plants were backcrossed to Cat100-1 inbred line or, in a few cases, they were crossed to L922 inbred line or F_1(Cat100-1 x L922).

R_2 progenies of six somaclones (CT963, CT971, CT986, CT1016, CT1021, CT1023) and R_1 to R_3 progenies of one somaclone (CT972) were analyzed for T-*urf13* PCR amplification and URF13 expression. Samples of 6-12 seeds from each generation were germinated for 5-6 days in autoclaved vermiculite, under dark conditions at 27 $\pm$1 ºC. Coleoptile samples consisting of about 10 mg were collected, without destroying the remaining seedling tissues. These seedlings were grown in the dark for a few more days, and then leaves were collected for extraction of mitochondrial proteins.

DNA extraction and PCR (polymerase chain reaction) amplification. Total DNA was extracted from coleoptile samples of single plants. Samples were homogenized in 100 μL of extraction buffer (50 mM KCl, 10 mM Tris-HCl pH 8.0, 5 mM EDTA, 1% Triton X-100, 1% carborundum (w/v), with a pestle (Kontes) in microcentrifuge tubes. Immediately, 100 μL phenol/chloroform (1:1) were added, and the extract was vortexed for 2-3 minutes. After full speed centrifugation in a microcentrifuge for 10 min., 120 μL supernatant was transferred to a new tube. DNA was precipitated with 12 μL sodium acetate 3 M and 105 μL isopropanol. After 10 min incubation at room temperature, DNA was sedimented in a microcentrifuge at full speed, and then it was resuspended in 25 μL TE buffer consisting of 10 mM Tris HCl pH 8.0, 1 mM EDTA (Sambrook *et al*., 1989). DNA samples of individual plants were used for PCR amplification in a Peltier thermal cyclers (PTC-100HB-60, MJ Research Inc.) as follows: 1 min. at 94 ºC, 2 min. at 55 ºC, 3 min. at 72 ºC (35 x); 5 min. at 94 ºC (1x). PCR reaction mixture consisted of 20 mM Tris-HCl pH 8.4, 50 mM KCl, 5 mM MgCl2, 1 mM each dNTP, 2 pmoles of each primer, 0.35 U *Taq*-polymerase, 2 μL DNA extract, water up to a final volume of 10 μL. PCR primers consisted of sequences annealing at the T-*urf13* flanking regions, immediately adjacent to the ATG start code ($^{5'}$CGG GAT CCA GAA AGG GAG ACT TTG GTC CC$^{3'}$)

or adjacent to the TGA stop codon (5'CCG AAT TCA AGG AAA ACA CTT TTT CGT CA3') of the gene. PCR products were separated on 1% agarose gel electrophoresis in TAE pH 7.4 buffer (40 mM Tris-acetate, 1 mM EDTA, 1.14 mL/L acetic acid) containing 0.5 μg/mL ethidium bromide.

Protein extraction and URF13 immunodetection. Leaf samples from 6 to 12 dark-grown seedlings were combined within each progeny, and homogenized in 0.5 M Tris-base pH 7.5, 5 mM EDTA, 0.4 M sucrose, and 35 μl/100mL β-mercaptoethanol, with mortar and pestle, in ice bath. Crude mitochondria extract was obtained by sequential centrifugations in cold rotor (4 °C). Firstly, the leaf extract was centrifuged at 3,000 x g for 10 min., followed by supernatant centrifugations at 3,000 x g for 10 min., and at 17,000 x g for 15 min. Then, the pellet was resuspended in buffer, and the extract was again centrifuged at 3,000 x g for 10 min., and at 17,000 x g for 15 min. The pellet was resuspended in 50-300 μL TE buffer (10 mM Tris-HCl pH7.5, 0.5 mM EDTA). Proteins were quantified by the Bradford method. Mitochondrial enriched extract samples containing 30 μg protein were fractionated by 15% SDS-PAGE and transferred to nitrocellulose membranes. A monoclonal antibody (MabURF13), gently provided by Dr. C. S. Levings III, was used for URF13 immunodetection. Antibody binding was detected with alkaline-phosphatase-conjugated anti-mouse as secondary antibody, according to standard procedures (Harlow and Lane, 1988).

Results

PCR amplification and URF13 immunodetection revealed diversity for T-*urf13* sequence in progenies of cms-T somaclones revertant to male fertility (Tables 1-2). Plants of five revertant somaclones, CT963, CT986, CT1016, CT1021, and CT1023, were analyzed within the R_2 progeny, and plants of somaclone CT972 were analyzed within R_1 to R_3. A stable male-sterile somaclone (CT971), plus Cat100-1 original cms-T and Cat100-1 normal (N) maize inbred line were used as controls. Figure 1 represents the URF13 protein detected by MabURF13 monoclonal antibody. Amplification of T-*urf13* sequence was detected as a fragment of about 400 bp, and it is illustrated in Figure 2. No alteration in protein size (13 kDa) and T-*urf13* PCR amplified fragment were observed among somaclones and controls. No T-*urf13* amplification or URF13 immunodetection was ever found in controls consisting of plants with N-cytoplasm.

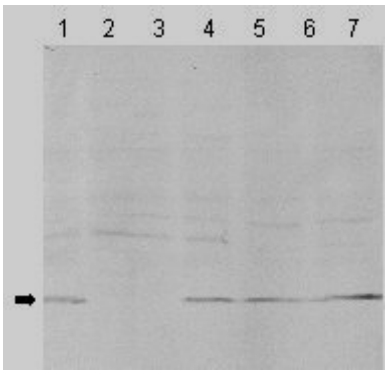

Figure 1. SDS-PAGE immunoblot of mitochondrial proteins from seedlings of maize inbreds and cms-T somaclones derived from methomyl-resistant callus F_1(Cat100-1cms-T x T204). Blots were probed with a URF13 monoclonal antibody that recognizes the carboxyl region of URF13 protein (arrow). Lane 1, Cat100-1cms-T; Lane 2, Cat100-1N; Lane 3, L922N; Lane 4, CT972-1-3-2cms-T; Lane 5, CT972-1-3-3cms-T; Lane 6, CT972-1-3-4cms-T; Lane 7, CT972-1-3-5cms-T

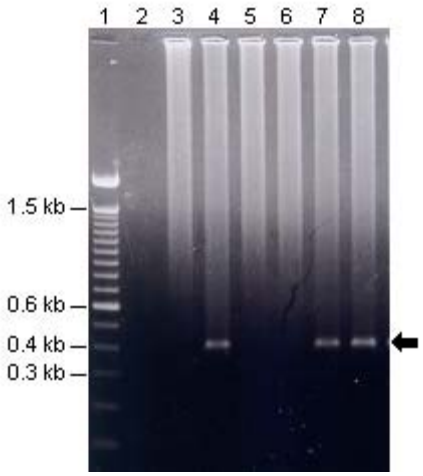

Figure 2. PCR amplified T-*urf13* DNA fragments of about 400 bp (arrow) from seedlings of maize inbred lines and cms-T somaclones derived from methomyl-resistant callus F_1(Cat100-1cms-T x T204), separated in agarose gel electrophoresis. Lane 1, 100-bp DNA ladder Molecular Markers; Lane 2, PCR reaction mixture without DNA; Lane 3, Cat100-1N; Lane 4, Cat100-1cms-T; Lanes 5-6, R_2 progeny of CT1021-1-2 cms-T revertant somaclone; note absence of DNA amplified fragments; Lanes 7-8, R_2 progeny of CT1021-1-2 cms-T revertant somaclone; note T-*urf13* amplified fragment

Somaclones CT1021 and CT1023 R_0 plants were fertile, and they were derived from the same callus line (23.6). CT1021 R_0 plant was crossed with Cat100-1, and generated 24 fertile plants. CT1023 R_0 plant was crossed with L922, generating 30 fertile plants plus one partial male sterile plant. URF13 protein was not detected in any of the analyzed R_2 seedlings derived from these two somaclones.

However, T-*urf13* sequence was amplified in 7 out of 10 seedlings from the R_2 progeny CT1021-1-2, although URF13 protein was not present in these seedlings (Table 1).

Table 1. Presence (+) and absence (-) of immunodetected URF13 protein, and of the PCR amplified T-*urf13* mitochondrial gene, in R_2 seedlings of several maize somaclones derived from methomyl-resistant cms-T callus [F_1(Cat110-1cms-T x T204)]

Somaclone	R_1 Parent[a]	R_1 Fertility	URF13 Protein in Groups[b] of R_2 Seedlings	Nº of R_2 Seedlings with T-*urf13*	
				Present	Absent
	CT1021-1-1	F	–		
	CT1021-1-2	F	–	7	3
CT1021-1	CT1021-1-3	F	–		
	CT1021-1-4	F	–		
	CT1021-1-5	F	–	0	10
	CT1023-1-1	P	–		
	CT1023-1-2	F	–		
	CT1023-1-3	F	–		
CT1023-1	CT1023-1-4	F	–	0	17
	CT1023-1-5	F	–		
	CT1023-1-6	F	–	0	15
	CT971-1-1	CMS	+	15	0
	CT971-1-2	CMS	+	11	0
CT971-1	CT971-1-3	CMS	+		
	CT971-1-4	CMS	+		
	CT971-1-5	CMS	+		
	CT1016-1-1	CMS	+	2	4
CT1016-1	CT1016-1-2	P	+	14	0
	CT1016-1-3	CMS	+		
	CT986-1-1	CMS	+		
	CT986-1-2	CMS	+		
CT986-1	CT986-1-3	CMS	+		
	CT986-1-4	CMS	+	15	0
	CT986-1-5	P	+		
	CT986-1-6	F	+	4	1
	CT963-1-1	CMS	+		
	CT963-1-2	CMS	+	16	0
	CT963-1-3	CMS	+		
	CT963-1-4	CMS	+		
CT963-1	CT963-1-5	CMS	+	6	0
	CT963-1-6	P	+	5	1
	CT963-1-7	CMS	+	10	0
	CT963-1-9	CMS	+	5	1
	CT963-1-10	CMS	+		
	CT963-1-11	P	+		

a. F = Fertile; P = Partial reversion to male fertility; CMS = Male-Sterile; ***b***. Protein samples extracted from groups consisting of at least six seedlings.

CT1016-1 was a partial male-fertile R_0 plant, CT986-1 and CT972 were cms-T, and CT963 developed terminal year with no tassel. All these four somaclones produced progenies of mainly cms-T plants, plus plants exhibiting total and/or partial male fertility. As shown in Tables 1 and 2, T-*urf13* fragment was amplified in individual samples from the majority of plants within progenies derived from these four somaclones. However, no amplification was obtained in many sister plants within the same R_2 progeny. As shown in Table 2, absence of T-*urf13* mitochondrial gene was also detected in R_3 plants of CT972. URF13 protein was detected in mitochondrial protein samples of all these four revertant somaclones. Unexpectedly, several fertile R_1 plants generated cms-T plants. Fertile R_1 plants of CT986 and CT972 produced plants containing T-*urf13* gene and expressing URF13 protein (Tables 1-2). For instance, CT986-1-6 was a R_1 fertile plant which generated 12 cms-T plus 3 partial fertile plants, and out of 5 analyzed plants 4 had T-*urf13* amplified by PCR. On the other hand, CT1016-1-1 and CT963-1-9 were cms-T R_1 plants that did not transmit T-*urf13* gene to several descendents. Furthermore, it was observed that among R_1 partial male-fertile plants of several somaclones there was a tendency of generating R_2 plants expressing URF13 protein and amplifying T-*urf13* sequence. CT1016-1-2 was a partial fertile R1 plant and produced 16 cms-T, 2 fertile, and 3 partially fertile R_2 plants in the field. Analysis of 14 R_2 sister seedlings revealed the presence of T-*urf13* sequence and URF13 expression in all these plants.

Table 2. Presence (+) and absence (-) of immunodetected URF13 protein and PCR amplified T-*urf13* mitochondrial gene, in R_1-R_3 seedlings of CT972 maize somaclone, regenerated from methomyl-resistant cms-T callus [F_1 (Cat110-1*cms*-T x T204)]

Progenies			Progenies Analyzed	URF13 Protein in Groups of Seedlings[b]	Nº of Seedlings with T-*urf13* Gene	
Somaclone	Female Parent	Fertility[a]			Present	Absent
CT972-1	R_0	CMS	R_1	+	5	1
CT972-1-1	R_1	F	R_2	+	6	0
CT972-1-2	R_1	F	R_2	+	6	0
CT972-1-3	R_1	F	R_2	+	6	0
CT972-1-1-1	R_2	CMS	R_3	+	6	0
CT972-1-1-2	R_2	CMS	R_3	+	6	0
CT972-1-1-3	R_2	CMS	R_3	+	5	0
CT972-1-1-4	R_2	F	R_3	+	6	0
CT972-1-1-5	R_2	F	R_3	+	6	0
CT972-1-1-6	R_2	F	R_3	+	6	0
CT972-1-1-7	R_2	P	R_3	+	6	0
CT972-1-1-8	R_2	P	R_3	+	6	0
CT972-1-1-9	R_2	P	R_3	+	4	0
CT972-1-2-1	R_2	CMS	R_3	+	1	3
CT972-1-2-2	R_2	CMS	R_3	+	5	1
CT972-1-2-3	R_2	CMS	R_3	+	4	2
CT972-1-2-4	R_2	F	R_3	+	5	0
CT972-1-2-5	R_2	F	R_3	+	4	1
CT972-1-3-1	R_2	F	R_3	+	4	2
CT972-1-3-2	R_2	CMS	R_3	+	6	0
CT972-1-3-3	R_2	CMS	R_3	+	5	1
CT972-1-3-4	R_2	CMS	R_3	+	5	1
CT972-1-3-5	R_2	CMS	R_3	+	5	1

a. F = Fertile; P = Partial reversion to male fertility; CMS = Male-Sterile; ***b***. Protein samples extracted from groups consisting of at least six seedlings

Sublines derived from somaclone CT972 showed that this cms-T R_0 somaclone generated 39 cms-T and 6 fertile R_1 plants. A total of 3 out of the 6 fertile plants were planted in the field and generated mainly cms-T, plus a few partially male-fertile and male-fertile R_2 plants. In all generations derived from CT972 cms-T somaclone, there was a tendency towards the presence of T-*urf13*, but this gene was absent in several R_2 and R_3 plants. Therefore, it seems that these plants produced gametophytic cells deficient in this mitochondrial

gene. On the other hand, as shown in Table 2, the T-*urf13* gene was present in male-fertile plants, but probably URF13 protein was not expressed in sufficient quantities to determine male sterility. These plants were apparently chimerical for the presence of this gene, which was amplified by PCR in DNA samples extracted from these plants, and transmitted to their descendants. CT972-1-1 produced cms-T, partially fertile, and R_3 fertile plants; however, all analyzed plants contained T-*urf13* sequence and expressed URF13. R_3 progenies derived from CT972-1-2 and CT972-1-3 were cms-T and fertile. URF13 protein was immunodetected in R_3 samples extracted from bulks consisting of 6 plants, but not every individual plant showed T-*urf13* sequence amplification. R_2 fertile plants transmitted T-*urf13* to the descendents, but this gene was lost in several of their R_3 descendants.

Discussion

Cms-T mitochondrial DNA rearrangements. The results of all revertant maize cms-T somaclones studied demonstrated that the T-*urf13* gene can exist in heteroplasmic state in plants of T-cytoplasm mutants derived from methomyl resistant callus. Within progenies of revertant somaclones diversity for T-*urf13* PCR amplification was detected, which could be explained by deletion of this mitochondrial gene. No diversity was found in the controls consisting of the stable male-sterile CT971-1 and the original Cat100-1cms-T inbred line, demonstrating that no amplification of T-*urf13* sequence was restricted to tissue culture-derived progenies containing partially or totally male-fertile plants. Therefore, T-*urf13* deletion was apparently created in T-mitochondria under tissue culture conditions, and transmitted to regenerated plants. The occurrence of several types of T-*urf13* alterations, including deletion, has been found within the mitochondrial DNA population of cms-T callus tissues derived from immature embryos of the American maize line A188 (Prioli L. M. *et al*., unpublished results). In maize with normal cytoplasm, heteroplasmy for a mutation in the mitochondrial cytochrome oxidase subunit II gene of calli and suspension cells (Wintz, 1994) has been described.

In contrast with a high stability of T-*urf13* in plants grown in the field, the cms-T mtDNA seems to have a tendency to recombine in cells grown in tissue culture, resulting in deletion of T-*urf13*. Elimination of T-*urf13* sequence in the cms-T mitochondrial genome has been previously reported in tissue culture-derived plants (Gengenbach *et al*., 1981; Abbott and Fauron 1986; Prioli *et al*., 1994). T-*urf13* is located in an unusual 3547-nucleotide mitochondrial DNA sequence, that contains two open reading frames, *T-urf13* and *orf221*, encoding proteins (Dewey *et al.*, 1986, 1987; Prioli *et al.*, 1993). The normal type and cms-T maize mitochondrial genomes are 570 kb and 540 kb in length respectively. The two maize types are very different in their genomic organization. The two genomes have 500 kb of common sequences, but there is considerable variation in sequence organization which may be due to recombinational events (Fauron and Havlik, 1989; Fauron *et al*., 1989b). DNA analysis of a revertant cms-T fertile somaclone (V3), after several generations of self-pollination, demonstrated T-*urf13* deletion associated with a genomic rearrangement involving mtDNA sequences. The authors concluded that recombinational events led to genomic reorganization, which eliminated some small sequences, including the T-*urf13* sequence, and duplicated some of the other sequences in this somaclone (Fauron *et al*., 1989a; 1990). However, mtDNA recombinants showing intermediate rearrangements leading to T-*urf13* elimination were not found in the V3 stable fertile somaclone. In the present work, lack of amplification of T-*urf13* was restricted to somaclones that produced descendents with partial or total reversion to male fertility, reinforcing the association of T-*urf13* and male sterility determined by the T-cytoplasm. These revertant somaclones seem to have mtDNA rearrangements with presence and absence of T-*urf13*; therefore, they are of interest to study cms-T genomic reorganization occurring under tissue culture conditions.

URF13 polypeptide and male sterility. As expected, amplification of T-*urf13* sequence via PCR was associated with URF13 expression, with exception of somaclone CT1021-1-2. Transcripts of T-*urf13* gene and URF13 protein expression have been extensively characterized in previous works (Dewey *et al*., 1986, 1988; Levings III, 1990; Huang *et al*., 1990; Ward and Levings III, 1991; Korth and Levings III, 1993). The absence of URF13 protein in the somaclone CT1021-1-2, in spite of T-*urf13* amplification, strongly suggests DNA sequence alteration leading either to no transcription of T-*urf13* gene or to an alteration of URF13 protein. A cms-T male fertile revertant mutant type (T4), which has a nucleotide alteration in the T-*urf13* sequence, was previously described in other maize lines (Wise *et al.*, 1987b; Prioli *et al*., 1994). This

biologically inactive mutant contains a 5-base-pair insertion in the T-*urf13* sequence, which results in a frameshift that generates a premature stop codon, truncating the predicted polypeptide at 8.3 kDa. This premature URF13 termination eliminates the antigenic site of all antibodies that have been raised against this protein. T-*urf13* nucleotide sequencing of CT1021-1-2 somaclone (R_2 plants) would be a suitable approach to better characterize male fertility reversion in this mutant. Creation of T-*urf13* variability through tissue culture seems to provide mutants that could be of interest to determine if male sterility and methomyl/toxin sensitivity can be dissociated in plants carrying the Texas cytoplasm. Although there is no doubt that URF13 protein is responsible for methomyl/toxin sensitivity, there are no conclusive experimental data demonstrating that the URF13 polypeptide is directly responsible for pollen abortion (Levings III, 1990). The results obtained in this work corroborate the evidences demonstrating that T-*urf13* is indeed directly associated with the cytoplasmic male sterility trait in plants with T-cytoplasm.

Heteroplasmy in Cms-T somaclones. The diversity for T-urf13 amplification obtained for all six revertant somaclones studied may be explained by heteroplasmy in this gene. Even very low copy numbers of T-urf13 could be detected in these somaclones by using a sensitive technique such as PCR. It is conceivable to interpret the T-urf13 presence and absence in diverse sister plants of the R_2 CT1021-1-2 as an evidence of heteroplasmy within the ear tissues of their parental somaclone. Some regions of the ear could consist of cells containing the T-urf13 mutated sequence, while cells in other regions could totally or partially lack this gene. Mitochondria containing the T-urf13 sequence could be transmitted to some descendants, while other descendants would not inherit this gene. The fact that one R_1 plant produced progeny containing T-urf13, and its four R_1 sister plants did not transmit the gene, indicates the possibility of heteroplasmy within the ear of the R_0 callus-regenerated plant. No detection of URF13 protein in R_2 CT1023-1-1 seedlings, which were descendants of a R_1 partially fertile plant, is an indication of heteroplasmy in the tassel tissues, that apparently did not occur in ear tissues. PCR amplification of T-urf13 from DNA samples from isolated tassel or ear sectors might demonstrate differences for the presence of this gene and confirm heteroplasmy.

All the other four revertant somaclones, including those derived from totally or partially male-fertile R_1 parent, expressed URF13 and several individuals of these progenies had the T-*urf13* gene amplified by PCR. These results reinforce the assumption of occurrence of heteroplasmy in the T-*urf13* gene in the callus-derived plants. The fact that a somaclone produces male sterile and fertile R_1 plants is enough to indicate heteroplasmy in ear tissues. In additon, the presence of T-*urf13* in R_2 seedlings derived from a fertile female parent is an evidence of diversity within the mtDNA population of the same plant, which probably had low T-*urf13* copy number and did not produce enough URF13 protein to cause pollen abortion.

The differences in PCR amplification in three generations (R_1-R_3) of somaclone CT972 descendants demonstrated that T-*urf13* gene may be maintained in heteroplasmic state through several generations, persisting in reproductive tissues and cells. Moreover, in the analysis of this somaclone and its progenies suggests occurrence of heteroplasmy within plant tissues, including ear tissues. Heteroplasmic state of T-*urf13* the plant is indicated by the fact that the R_0 male sterile parent produced six fertile R_1 plants, which then produced R_2 and R_3 plants containing this gene.

For many years it has been assumed that the vast majority of mitochondrial genomes of a single individual are identical, both in the same tissue and within different tissues. However, heteroplasmy has been described in humans and animals (Asley *et al*., 1989; Comas *et al*., 1995; Grzybowski, 2000). In plants, it was recently reported in a maize yellow striped line derived from tissue culture, that is a mutant for the mitochondrial *coxII* gene (Marienfeld *et al.*, 1993; Wintz, 1994). The heteroplasmy for the presence of *T-urf13* sequence and URF13 expression in the tissue culture-derived plants and their progenies identified in the present work is apparently the cause of the reversion to male fertility in these plants. These somaclones are rare mitochondrial genotypes suitable to study molecular recombination mechanisms of plant mitochondrial genomes.

The mitochondrial variability created in maize cms-T callus, and their regenerated plants and progenies, represent a valuable model system to study reversion to male fertility, mtDNA rearrangements leading to a selective elimination of T-*urf13* gene, association of Texas male sterility and fungal toxin sensitivity, and mitochondrial heteroplasmy in plants.

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