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Chloroplast DNA polymorphism in fertile and male-sterile cytoplasms of sorghum (*Sorghum bicolor* (L.) Moench)*

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Summary. Restriction endonuclease patterns of chloroplast DNA (cpDNA) were consistently distinguishable between fertile and male-sterile cytoplasms of sorghum [*Sorghum bicolor* (L.) Moench], whereas no differences in restriction patterns of cpDNA among male-sterile (A_1) lines, including six isocytoplasmic strains, were revealed in this study. It is suggested that chloroplast DNA may contribute to the male sterility of A_1 lines used currently in hybrid sorghum production.

Key words: Chloroplast DNA – *Sorghum bicolor* (L.) Moench – Male-sterile cytoplasm

Introduction

Chloroplast DNA (cpDNA) is a specific circular molecule ranging in size from 120 kb to 217 kb in angiosperms (Palmer 1985). Although cpDNA or mitochondrial DNA (mtDNA) from a given species is of a unique size, the highly uniform size of cpDNA suggests that selection maintains a fairly restricted chloroplast genome size. Alterations in mtDNA have been related to cytoplasmic male sterility (cms) of maize (Levings and Pring 1976; Pring and Levings 1978), sorghum (Pring et al. 1982; Conde et al. 1982; Lee et al. 1989), sugarbeet (Powling 1982), and pearl millet (Smith and Chowdhury 1989). The contribution of cpDNA to male sterility cannot be entirely excluded; the genetic basis of male sterility is probably related to cpDNA in tobacco (Frankel et al. 1979) and cotton (Galau and Wilkins 1989). Pring et al. (1982) postulated that the widely used A_1 male-sterile (milo) cytoplasm of sorghum contains different mtDNA than normal fertile (kafir) cytoplasm. However, the contribution of cpDNA changes to male sterility in sorghum is still uncertain. The purpose of the study reported here was to detect the alterations in restriction endonuclease patterns of cpDNA in various fertile and male-sterile cytoplasms of sorghum [*S. bicolor* (L.) Moench] and to provide a tool in the search for additional cytoplasmic male-sterile lines for hybrid sorghum production.

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Materials and methods

Plant materials

The plant material used in this study consisted of: six isocytoplasmic male-sterile lines KS34A through KS39A, which possess cytoplasms from *S. arundinaceum* (SA 1471 and PI 258806), *S. verticilliflorum*, *S. sudanense*, *S. conspicuum*, and *S. niloticum*, respectively (Ross and Hackerott 1972); their maintainer line, Combine Kafir 60B (CK60B); two fertile cultivars, 'Tx2772' and 'Tx2775'; and two pairs of grain sorghum, Redlan A and B and KS5 A and 5B.

Chloroplast DNA isolation

The chloroplast DNA isolation procedure was adapted from those of Mourad and Polacco (1989), Kolondner and Tewari (1975), Palmer (1986), and Rode et al. (1985). Seeds were germinated and seedlings were grown in a growth chamber or greenhouse for 2 to 3 weeks. Young healthy leaves were collected and rinsed in ice-cold distilled water. The leaves were cut into 1-cm sections, placed in a chilled Waring blender with approximately 4 ml buffer A (0.3 *M* mannitol, 50 m*M* TRIS-HCl, pH 8.0, 3 m*M* EDTA, 1 m*M* β-mercaptoethanol, and 0.1% BSA) per gram fresh leaves. The homogenate was filtered through four layers of cheesecloth and four layers of miracloth. The filtrate was centrifuged at 3,000 rpm in a Sorvall centrifuge for 7 min. The crude chloroplast pellet was dispersed in buffer B (0.3 *M* mannitol, 50 m*M* TRIS-HCl, pH 8.0, and 1 m*M* EDTA), cen-

trifuged at 3,000 rpm, resuspended again, and treated with DNase I (100 μg/ml) in the presence of 0.02 *M* $MgCl_2$ at 0 °C for 1 h. The reaction was stopped by adding two volumes of buffer C (0.3 *M* mannitol, 50 m*M* TRIS-HCl, pH 8.0, and 100 m*M* EDTA). The chloroplast pellet obtained after centrifugation at 1,500 g for 10 min was washed four times with buffer D (0.3 *M* mannitol, 50 m*M* TRIS-HCl, pH 8.0, and 25 m*M* EDTA). The resulting chloroplast pellet was lysed in 2.5–3.0 ml lysis buffer (50 m*M* TRIS-HCl, pH 8.0, 20 m*M* EDTA, and 1% sarkosinate) in the presence of proteinase K (200 μg/ml) and 0.26 g/ml CsCl. After gentle stirring for 1 h at 37 °C, 3.0 g CsCl and 400 μl ethidium bromide (10 mg/ml) were added to the lysate, and ultracentrifugation (58,000 rpm) was performed in a Beckman VTi 65 rotor at 20 °C for 10–16 h. The DNA band was recovered using UV light, and ethidium bromide was removed by at least six extractions with TE-saturated *n*-butanol. cpDNA was precipitated by adding one-tenth volumes of 3 *M* NaOAC (pH 5.2) and two volumes of 100% ethanol at −20 °C and incubating overnight. The DNA was pelleted at 30,000 rpm for 1 h, washed with 70% ethanol, briefly dried in a vacuum desiccator, and dissolved in 50–100 μl TE buffer (10 m*M* TRIS-HCl, pH 8.0, 1 m*M* EDTA).

Digestion of chloroplast DNA

The restriction enzymes, HindIII, BamHI, PstI, and XhoI (Promega Inc), were used according to recommendations of the suppliers. Aliquots of 8–10 μg cpDNA were digested in 25 μl of reaction mixture with 20–40 units of the restriction enzyme and incubated at 37 °C for 12–20 h. The reaction was terminated by adding 2.5 μl 10 × loading dye (2.5% bromophenol blue, 0.25% xylene cyanol, and 25% Ficoll, type 400).

Agarose gel electrophoresis

Digested cpDNA was loaded onto a 25-cm-long, 0.8% agarose gel. Electrophoresis was carried out in TBE buffer (100 m*M* TRIS, 100 m*M* boric acid, and 2 m*M* EDTA, pH 8.3) at 40–50 volts for 20–24 h at room temperature. The gel was stained with 0.25 μg/ml ethidium bromide for 30 min with gentle agitation, placed on a UV transilluminator, and photographed with type 55 P/N Poloroid film.

Results

The restriction patterns of cpDNAs from six isocytoplasmic male-sterile lines and two fertile lines of sorghum after digestion with HindIII are shown in Fig. 1. Chloroplast DNA from six male-sterile lines showed the same restriction pattern, although these lines have different cytoplasmic sources. Two male fertile lines, Tx2775 and CK60B, possessed a band at approximately 3.8 kb on the 0.8% gel, which is different from the band that appeared at approximately 3.7 kb in the restriction patterns of six male-sterile sorghum. The same results were obtained after HindIII restriction endonuclease digestion of cpDNA from two pairs of grain sorghum inbreds, Redlan A and Redlan B and KS5A and KS5B (Fig. 2).

The restriction enzyme patterns of cpDNA from Tx2772 and KS36A digested with BamHI are shown in Fig. 3. The patterns show that the male-fertile line

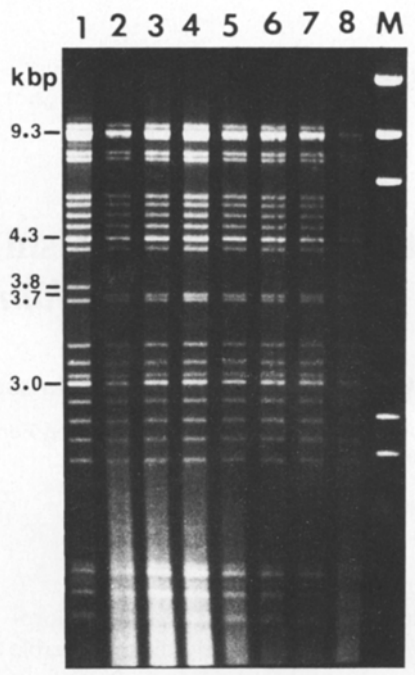

Fig. 1. Agarose gel electrophoretic patterns of HindIII digestion of cpDNA from fertile and six cytoplasmic male-sterile lines of sorghum. *Lane 1* Tx2775; *lane 2* KS34A; *lane 3* KS35A; *lane 4* KS36A; *lane 5* KS37A; *lane 6* KS38A; *lane 7* KS39A; *lane 8* CK60B; *M* lambda DNA marker. The molecular weights were estimated from the lambda DNA markers. The same markers were used for all other figures and tables

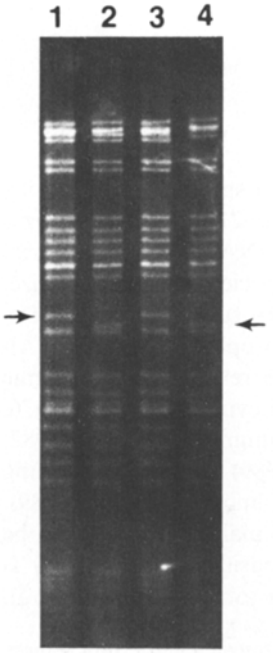

Fig. 2. Agarose gel electrophoretic patterns of HindIII digestion of cpDNA from fertile and male-sterile counterparts of sorghum. *Lane 1* KS5B; *lane 2* KS5A; *lane 3* Redlan B; *lane 4* Redlan A. *Arrows* indicate the difference in cpDNA fragments

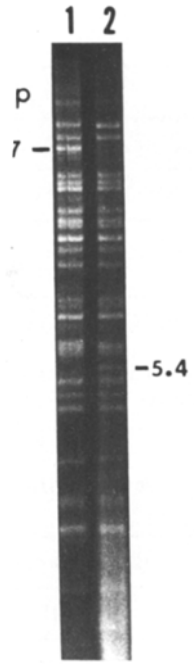

Fig. 3. Agarose gel electrophoretic patterns of BamHI digested cpDNA from fertile and male-sterile sorghum. *Lane 1* Tx2772 (fertile); *lane 2* KS36A (male sterile)

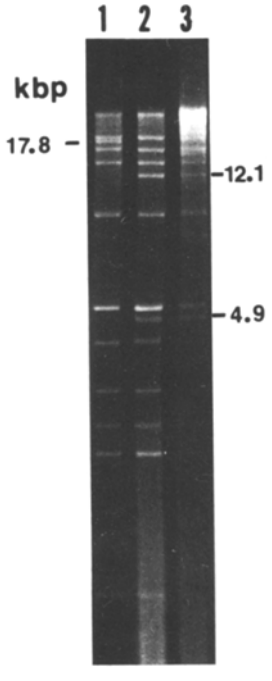

Fig. 4. Agarose gel electrophoretic patterns of PstI-digested cpDNA from fertile and male-sterile sorghum. *Lane 1* Tx2772; *lane 2* KS34A; *lane 3* KS36A

Tx2772 had a 10.7 kb BamHI fragment that was missing in the male-sterile line KS36A. Instead, KS36A possessed a 5.4 kb band that was not present in the fertile line. In the PstI digestion of cpDNA, the 17.8 kb band present in the fertile line (Tx2772) was replaced by bands of 12.1

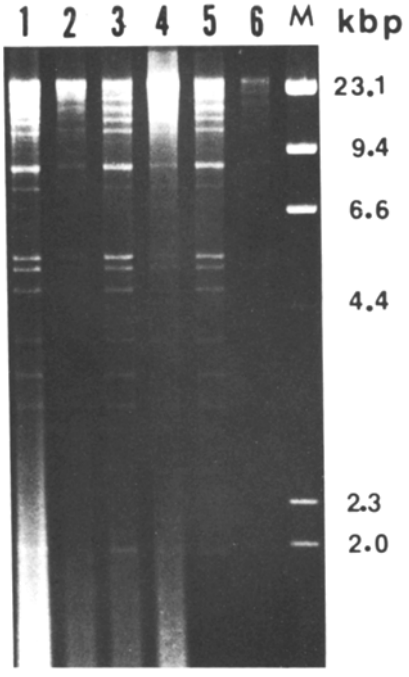

Fig. 5. Agarose gel electrophoretic patterns of PstI-digested cpDNA of six isocytoplasmic lines of male-sterile sorghum. *Lane 1* KS34A; *lane 2* KS35A; *lane 3* KS36A; *lane 4* KS37A; *lane 5* KS38A; *lane 6* KS39A; *M* lambda DNA marker

and 4.9 kb in the two male-sterile lines (KS34A and KS36A) (Fig. 4). It is noteworthy that the two bands in the male-sterile lines add up to the size of the 17.8 kb band in the fertile line. Differences between fertile and male-sterile lines also could be identified in XhoI patterns: KS34A, KS35A, and KS36A had unique 6.4 and 3.9 kb bands, whereas Tx2772 had 7.3 and 3.4 kb bands instead (data are not shown).

The restriction endonuclease patterns of PstI-digisted cpDNAs from six isocytoplasmic male-sterile lines were also the same (Fig. 5). In addition, no differences between the XhoI restriction patterns of KS34A and KS36A were detected (Fig. 6).

Table 1 summarizes the results of differences in restriction fragments of cpDNA from fertile and male-sterile lines of sorghum. It should be noted that the fluorescence intensities of the HindIII fragments with estimated sizes of 9.3 kb, 4.3 kb, and 3.0 kb (Fig. 1; Table 2) and of the PstI fragments of 5.2 kb and 3.0 kb (Fig. 4; Table 2) were much higher than those of their larger neighboring fragments, which implies that these cpDNA bands contained two or more fragments of similar molecular size.

Discussion

Diversity in chloroplast DNA was apparent in the fertile and cytoplasmic male-sterile lines of grain sorghum com-

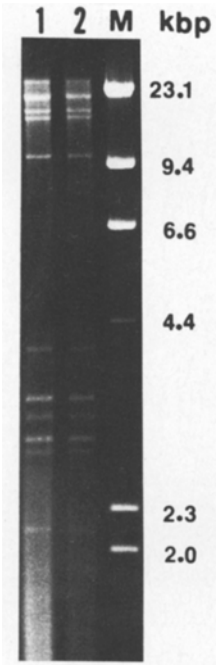

Fig. 6. Agarose gel electrophoretic patterns of XhoI-digested cpDNA from two isocytoplasmic lines of male-sterile sorghum. *Lane 1* KS34A; *lane 2* KS36A; *M* lambda DNA marker

pared in this study. All male-sterile lines exhibited differences in restriction enzyme digestion patterns when compared to the fertile lines. The variation was detected at the cleavage sites of the four restriction enzymes tested (BamHI, HindIII, PstI, and XhoI) (Table 1). On the contrary, the polymorphism of cpDNA restriction patterns was not found among male-sterile lines with different cytoplasms. Restriction endonuclease patterns among Redlan A, KS5A, and KS34A through KS39A were indistinguishable using several enzymes, suggesting that there are no differences in these restriction sites for cpDNAs between milo cytoplasm and the cytoplasms of KS34A through KS39A, even though the two groups were from different sources (Ross and Hackerott 1972). It is known that chloroplast genomes of higher plants have been conserved during evolution and are maternally inherited (Palmer et al. 1983; Palmer 1985; Vedel et al. 1981). Previous studies have indicated that variation occurs in mtDNA restriction enzyme patterns between fertile and male-sterile lines of sorghum (Conde et al. 1982; Lee et al. 1989). Because differences in cpDNA restriction sites between fertile and cytoplasmic male-sterile sorghum have been consistently observed, a cpDNA contribution to the male sterility of sorghum species cannot be excluded. Further research involving the characterization of specifically identified fragments of cpDNA and their effects on cytoplasmic male sterility in sorghum and comparisons of A_1 and other male sterility-inducing cytoplasms not utilized in this study will help resolve this issue.

Table 1. Polymorphism of restriction fragments of cpDNA from fertile and male-sterile cytoplasms of sorghum

Restriction fragments (kbp)							
HindIII		BamHI		PstI		XhoI	
N[a]	CMS[b]	N	CMS	N	CMS	N	CMS
3.8	3.7	10.7	5.4	17.8	12.1 4.9	7.3 3.4	6.4 3.9

[a] cpDNA from normal or fertile sorghum: CK60B, Tx2772, Tx2775, KS5B, or Redlan B
[b] cpDNA from cytoplasmic male-sterile (cms) lines of sorghum: KS34A through KS39A, KS5A or Redlan A

Table 2. Estimation of cpDNA molecular size of HindIII and PstI fragments in fertile sorghum

	cpDNA fragments (kbp) of sorghum	
	HindIII	PstI
	12.4	26.9
	9.3[a]	18.9
	9.1	17.8
	8.9	16.0
	7.5	13.5
	7.1	8.9
	5.3	5.2[a]
	5.0	4.5
	4.7	3.7
	4.5	3.3
	4.3[a]	3.0[a]
	4.2	2.0
	3.8	
	3.7	
	3.4	
	3.2	
	3.1	
	3.0[a]	
	2.7	
	2.4	
	2.0	
	1.6	
	1.2	
	1.0	
	0.9	
	0.8	
Total	131.7	131.9

[a] Doublets

The present data also imply that there are two possible causes of the differences in the restriction patterns of the cpDNA from fertile and male-sterile sorghum. One is the deletion of a cpDNA HindIII or BamHI fragment in male-sterile lines compared to fertile lines (Figs. 1, 2, 3; Table 1). The other is the split of a fragment (PstI) or two fragments (XhoI) into the two new fragments in the

male-sterile lines that add up to a similar molecular size in fertile sorghum (Fig. 4; Table 1). More details could be obtained if the different fragments from fertile and male-sterile sorghum were labeled and used as markers to blot the cpDNA digested with these restriction endonucleases.

The HindIII and PstI restriction patterns suggest that certain bands were present in multiple (probably two) copies, including two fragments of similar molecular sizes, as evidenced by their fluorescence intensities. Nevertheless, the molecular sizes of cpDNA from sorghum and maize have been estimated to lie between 136-140 kb (Dang and Pring 1986). The size of the sorghum cpDNA digested with HindIII and PstI in this study was estimated to be 131.7 kb and 131.9 kb, respectively, by assuming that the 9.3 kb, 4.3 kb, and 3.0 kb Hind III fragments and the 5.2 kb and 3.0 kb PstI fragments were doublets. The molecular size of the cpDNA tended to be much smaller without making a correction for these fragments (Figs. 1 and 4; Table 2). Such cpDNA repeats are known in other higher plants such as maize (Doebley et al. 1987), wheat (Bowman et al. 1981), and legumes (Palmer et al. 1983). The repeats could have an influence on the evolution of chloroplast genomes. The origin, distribution, and function of chloroplast DNA repeats in sorghum species needs to be further evaluated.

It is concluded that the fertile and male-sterile cytoplasms currently used in sorghum production could be distinguished by any one of the restriction enzymes used in this study (Table 1). Using several restriction enzymes that produce multiple fragments, we were unable to detect any significant differences in chloroplast DNA restriction patterns either among various male-sterile cytoplasms or among the fertile cytoplasms. The occurrence of repeats or fragments of a similar molecular size in the sorghum chloroplast genome needs additional study to clarify their function and significance.

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