RFLP Pattern of Mitochondrial DNA in Pearl Millet

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Introduction

Mitochondrial genome variation, and interactions of the mitochondrial and nuclear genomes, have been implicated in the cytoplasmic-nuclear male-sterility systems available for commercial hybrid seed multiplication in many crop species (Leaver et al. 1988). Molecular characterization of plant mitochondrial genomes typically requires isolation of mitochondrial DNA (mtDNA) from young seedlings following protocols that efficiently exclude nuclear DNA and RNA by DNAse and RNAse treatments followed by differential centrifugation to isolate mitochondria. The maternally inherited cytoplasmic genome, of which the mitochondrial genome is a part, constitutes a very small proportion of the total plant genome. Thus, large numbers of seedlings are required to meet experimental requirements for mtDNA studies. This is a constraint, particularly in highly cross-pollinated crops like pearl millet (*Pennisetum glaucum* (L.) R. Br.) where production of selfed/sibbed seed is much more expensive, tedious and time-consuming than that of open-pollinated (OP) seed.

While characterizing cytoplasmic-nuclear male sterility (CMS) sources in pearl millet, we faced problems in producing selfed or sibbed seed of several materials in quantities sufficient to give mtDNA for RFLP analysis. Alternative approaches with potential to provide sufficient DNA were evaluated. Use of OP seed instead of sibbed-seed was explored as both have the same cytoplasmic genome. We also explored use of total DNA (tDNA) for this purpose, as it is more quickly isolated, requires less seed, and is potentially more cost-effective than using mtDNA.

Plant material. In this study we used one male-fertile line (81B) and five iso-nuclear male-sterile lines (A-lines) with diverse CMS systems in the nuclear genetic-background of their common maintainer (81B). The A-lines include 81 A_1 having the A_1 cytoplasm (Burton 1965), 81A_2 and 81A_3 having the A_2 and A_3 cytoplasms (Burton and Athwal 1967), 81A_v having the cytoplasm of *P. glaucum* subsp. *violaceum* (Lam.) L. Rich. (Marchais and Pernes 1985), and 81A_4 = 81A_m having the cytoplasm of *P. glaucum* subsp. *monodii (=violaceum)* (Maire) Brunken (Hanna 1989). The isonuclear A-lines studied here were developed, or assembled from other sources, at ICRISAT (Rai et al. 1996).

Seed multiplication. Two types of seed lots were produced: open-pollinated and sibbed seed. Open-pollinated seed lots came from the bulk harvest of open-pollinated panicles of each line. Sibbed seed lots were produced by bagging the panicles of each line at the boot stage and pollinating them with 81B pollen at the time of stigma emergence. Panicles from each line were harvested separately and bulked. Care was taken to ensure that off-type plants and pollen shedders were excluded from seed production of each A-line for this study.

DNA probes. One homologous (pearl millet) and two heterologous (maize) mtDNA probes were used in the RFLP analysis. The homologous probe was a 4.7 kb fragment from *P*srI-digested mtDNA of A_1 pearl millet male-sterile cytoplasm, provided by RL Smith, University of Florida, USA (Smith and Chowdhury 1991). The two maize mtDNA probes were *atp6* and *coxI*. The F_1-F_0 ATPase subunit 6, *atp6* (Dewey et al. 1985), was provided by C.S. Levings III, Genetics Department, North Carolina State University, Raleigh, NC, USA, as purified plasmid DNA with this insert. A clone of cytochrome *c* oxidase subunit 1, *coxI* (Isaac et al. 1985) was provided by CJ Leaver, Department of Botany. University of Oxford, Oxford, UK.

The three probes were used to hybridize mtDNA/tDNA digested with three restriction enzymes in eight different combinations (Table 1).

DNA analysis. Previously described protocols were followed for mtDNA isolation, its digestion with *Bam*HI, *Hind*III and *Pst*I, separation of DNA fragments and their staining, Southern transfer, random-primed DNA probe labeling, hybridization, and autoradiography (Chhabra et al. 1998).

Table 1. Enzyme-probe combinations tested for detection of RFLP in mtDNA (mt) and tDNA (t) using open-pollinated (O) and sibbed-seed (S) of pearl millet male-fertile maintainer line 81B and five isonuclear cytoplasmic male-sterile lines ($81A_1$, $81A_2$, $81A_3$, $81A_4$ = $81A_m$ and $81A_v$).

Enzyme-probe combination	$81A_1$			$81A_2$			$81A_3$			$81A_4$			$81A_v$			81B	
	mt	t	O	S	mt	t	O	S	mt	t	O	S	mt	t	O	S	mt	t
*Hind*III-4.7 kb	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
*Hind*III-*coxI*	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+
*Pst*I-4.7 kb	-	-	-	-	-	-	+	+	-	-	+	+	-	-	+	+	-	-
*Pst*I-*atp6*	-	-	-	-	-	-	+	+	-	-	+	+	-	-	-	-	-	-
*Pst*I-*coxI*	-	-	-	-	-	-	+	+	-	-	+	+	-	-	-	-	-	-
*Bam*HI-4.7 kb	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
*Bam*HI-*atp6*	-	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-
*Bam*HI-*coxI*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ indicates that the particular enzyme-probe combination was tested.

Total DNA was isolated using the method of Dellaporta et al. (1983). Restriction enzyme digestion reactions were set up as per supplier's instructions with ~15 µg DNA in a final volume of 30 µL. Southern blotting, prehybridization, probe labeling, and hybridization procedures were the same as those used for the mtDNA samples.

Results and Discussion

RFLP banding patterns based on mtDNA for the OP seed were identical to those based on mtDNA from sibbed seed (not shown) with all 31 cytoplasm x enzyme-probe combinations tested (Table 1). This clearly indicated that OP and sibbed seed can be used of mtDNA-RFLP analysis with equal reliability.

In all four enzyme-probe combinations tested (Table 1), RFLP banding patterns were identical from tDNA and mtDNA Southern blots as illustrated for *Bam*HI-*coxI* in Figure 1. The only difference observed was that bands appeared hazy and their resolution was reduced when tDNA was used. A few bands with very similar molecular weights appeared fused, resulting in thick hazy bands when digested tDNA was used in place of digested mtDNA.

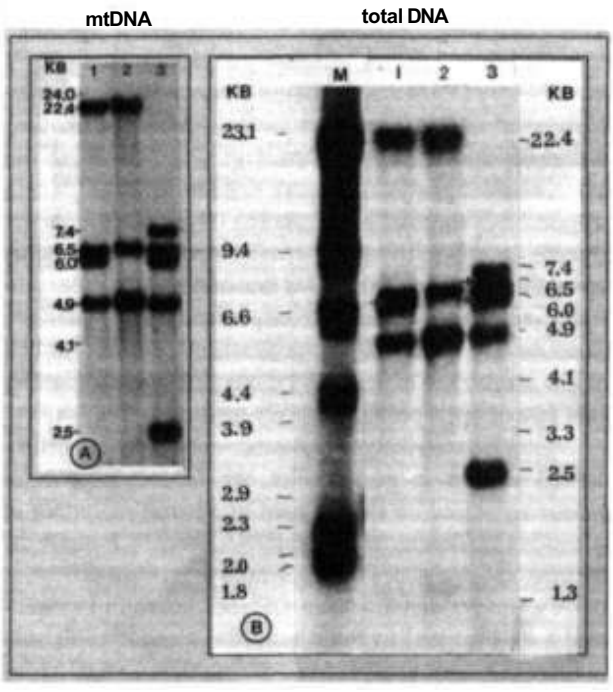

Figure 1. Southern blot hybridizations of tDNA (A) and mtDNA (B) of pearl millet male-fertile (81B) and two cytoplasmic male-sterile lines (81 A_1 and 81A_m= 81A_4). The tDNA and mtDNA samples were digested with *Hind*III and *Bam*HI, respectively, and probed with *cox*I. Fragment sizes are given in kilobases (kb). Arrows indicate apparent fusion of two (6.5 kb and 6.0 kb) or three (7.4 kb, 6.5 kb and 6.0 kb) fragments in tDNA Southern blots. Genotypes: B = 81B, A_1 = 81A_1, A_m = 81A_m = 81A_4.

For example, 6.5 kb and 6.0 kb bands in 81B merged to form a single thick band in *BamHI-coxI* Southern blots of tDNA (Fig. 1B), whereas, they appeared as independent distinct bands in mtDNA Southern blots (Fig. 1A). Similarly, three bands of 7.4 kb, 6.5 kb and 6.0 kb were not well resolved in tDNA blots (Fig. 1B). This might be because tDNA digested with restriction enzymes yields a larger number of fragments, which then require 1) longer gels, 2) shorter exposure times of autorads, or 3) use of a phospho-imager, in order to achieve effective separation of each fragment comparable to that possible for the smaller fragment numbers from digested mtDNA. The similarity in intensity of hybridization for the tDNA and mtDNA blots is indicative of the high specificity of probes that were used in this study. Results might not have been as favorable if less-specific probes had been used. The resolution in tDNA bands increased when longer gels were used for digested tDNA (data not shown). Our results indicate that mtDNA probes can be used with tDNA preparations for RFLP analysis of mitochondrial genome differences. However, this approach will need to be used with caution if repeated sequences are used as probes or copies of the mitochondrial genome are present in the nucleus.

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