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Molecular analysis of mitochondrial DNA of lines representing a specific CMS-fertility-restorer system of pearl millet [*Pennisetum glaucum* (L.) R. Br.] by RAPD markers

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Abstract

Molecular characterization of the mitochondrial DNA [mtDNA] from the etiolated pearl millet [*Pennisetum glaucum* (L.) R. Br.] seedlings of cytoplasmic male sterile (CMS) line having A_1 cytoplasm alongwith its maintainer, restorer and restored lines of hybrid RHRBH-8609 (*Shradha*) revealed polymorphism by 10 RAPD primers. Only four primers showed polymorphism between a CMS, RHRB-1A and its fertile isonuclear line, RHRB-1B. However, 5 primers showed polymorphism between the CMS line and its restorer line, RHRB-138R. The primers OPC15, OPC16 and OPG13 generated fragments of ~3.90 kb, 1.59 kb and 1.02 kb size only in the mtDNA of a male sterile line which might be associated with the CMS phenotype of RHRB-1A in pearl millet.

Key words: Pearl millet, cytoplasmic male sterility, mtDNA, RAPD markers.

Introduction

Cytoplasmic male sterility (CMS) is used extensively for commercial F_1 hybrid seed production in more than 150 crop species including pearl millet [*Pennisetum glaucum* (L.) R. Br.]. An increasing number of discoveries of new male sterility sources are being made, and the established sources of existing cytoplasms are being diversified by incorporating desirable nuclear genes [1]. Fertility restoration patterns of the hybrids through the use of common restorer lines have been traditionally used for CMS classification. However, it was shown that the nuclear genetic background of CMS lines influences the fertility restoration patterns of the hybrids [2]. This then requires the use of isonuclear 'B' lines for fertility restoration studies and classification of CMS sources. Development of isonuclear 'A' lines is both time- and resource-consuming [3]. Therefore, the identification of cytoplasmic molecular marker(s) is of importance to pearl millet breeders in differentiating CMS sources. It is also important to find out differences between the sterile and fertile cytoplasm in a particular nuclear background.

At the molecular level, attempts have been made in the past in various crops including pearl millet by extensively employing restriction fragment length polymorphism [RFLP] and hybridization patterns of mtDNA to categorize CMS sources and to distinguish the sterile lines from the fertile ones [4, 5]. However, this technique is time-consuming and needs a fairly large amount of pure mtDNA, a specific mt gene probe and its radiolabelling. On the contrary, RAPD analysis is an easy, quick and time-saving method which requires only minute quantities of crude DNA and random primers. Nakajima et al. [6] obtained more accurate and reliable results by RAPD analysis of mtDNA and genomic DNA of eight carrot varieties than those obtained from RFLP analysis. We found no published reports concerning characterization of mtDNA from the pearl millet CMS system by RAPD analysis. The segregation studies have shown the expected dominant nature of RAPD markers [7]. However, the RAPD technique has not yet been applied to organellar DNA of pearl millet and has not yet been investigated systematically. Hence, the present investigation was undertaken on molecular characterization of mtDNA of CMS and fertility restorer parental lines of RHRBH 8609 of pearl millet by RAPD analysis.

Materials and methods

Plant materials: The seeds of CMS RHRB 1A line having A_1 cytoplasmic source alongwith its maintainer RHRB 1B, restorer RHRB 138R and restored lines (*Shradha*), RHRBH 8609 developed at All India Co-ordinated Pearl Millet Improvement Project, Mahatma Phule Krishi Vidyapeeth, Rahuri were used in the present investigation. About 50g of seeds of each of these lines were surface sterilized with 0.1% $HgCl_2$ and allowed to germinate in the dark at 35°C in vermiculite. The etiolated shoot tissues (7 day old) were harvested, surface sterilized with ethanol, washed several times with sterile distilled water, blotted dry in filter paper folds and used for mtDNA isolation.

Isolation and purification of mtDNA: Isolation and purification of mtDNA was performed as per the methods described earlier by Munjal and Narayan [5] and Chase and Pring [8] with some modifications. Briefly, the method consists of grinding the shoot portions in a chilled mortar and pestle with three volumes of isolation buffer followed by differential centrifugation for pelleting the mitochondria.

PCR amplification of mtDNAs: RAPD analyses of mtDNA of the above four lines were carried out using a total of 44 random primers procured from Operon Technologies, California, USA. Mitochondrial DNA was amplified in a 25 µl volume consisting of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 100 µM each of dATP, dCTP, dGTP and dTTP (Bangalore Genei Pvt. Ltd., Bangalore, India), 0.2 µM random operon primers, 20 ng mtDNA and 0.5 U of *Taq* polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India). Polymerase chain reaction (PCR) amplification of mtDNA was performed in a thermocycler (PTC-100TM Programmable Thermal Controller Version 5.2, MJ Research Inc., USA). The thermocycler was operated for first cycle at 92°C for 4 min, 34°C for 1 min and 72°C for 2 min for denaturing, annealing and primer extension, and was programmed for 45 further cycles at 92°C for 30 sec, 34°C for 1 min and 72°C for 2 min. It was followed by the final extension step of 10 min at 72°C. Amplified mtDNA was electrophoresed on 1.5% (w/v) agarose gel, run in 1X TBE buffer [9], stained with ethidium bromide and the gels were photographed.

Data analysis: The RAPD gels were scored for presence or absence of the amplified fragments for distinguishing the differences between male sterile and fertile lines as described earlier by Samec and Nasinec [10].

Results and discussion

The RAPD analyses of the mtDNA of RHRB1A, 1B, 138R and RHRBH8609 were carried out by employing a total of 44 random primers. Two primers *viz.*, OP14 and OPI19 did not amplify mtDNA fragments of any of the above four lines. However with 13 primers - OPC3, OPC7, OPG3, OPG7, OPG11, OPG19, OPG20, OPI13, OPI14, OPI16, OPI17, OPK20 and OPL19 - the amplified products did not show uniform amplification. Seven primers *viz.*, OPG2, OPG18, OPK4, OPM17, OPM18, OPS3, and OPX14 amplified mtDNA with smearing. Twelve primers *viz.*, OPC13, OPG2, OPG6, OPG8, OPG9, OPG12, OPG19, OPI6, OPI18, OPI20, OPL14, OPL20 and OPG25 produced monomorphic bands. However, as shown in Figures 1-3, 10 primers *viz.*, OPC15, OPC16, OPG13, OPG14, OPG15, OPL17, OPK19, OPS4, OPS5 and OPS6 amplified reproducible polymorphic bands. The primers OPC16, OPG13, OPG17, OPK19, OPS4, OPS5 and OPS6, showed polymorphism ranging from 10-15%, while the OPC15 showed 6.00% polymorphism and OPG14 showed 8.57%. Further, it was observed that 10 random primers synthesized a total of 303 polymorphic bands, of which 32 showed the polymorphism of 10 per cent.

Only four primers showed differences in the fragments of CMS and maintainer lines. With primer OPC16, the amplicon of 3.90 kb appeared only in the mtDNA of the male sterile line, RHRB 1A but was absent in its isonuclear B line (Figs. 1, lanes 1 and 2). From Fig. 2, it was observed that the primer OPG17 synthesized the fragments of 4.17 kb and 3.96 kb (lane 2), whereas OPG15 synthesized a 3.70 kb fragment (lane 6) which appeared only in the B line and was absent in the mtDNA of the CMS line. With a primer OPS5, a fragment of the size of 2.43 kb (Fig. 3, lane 9) was present in the CMS line and was absent in the maintainer line, whereas a fragment of 1.34 kb (Fig. 3, lane 10) was present in the maintainer line and was absent in the CMS line.

Marked differences were also observed in respect of presence or absence of bands in the male sterile, A and restorer, R line. It was observed that five primers *viz.*, OPG14, OPG17, OPK19, OPS4 and OPS5 showed differences in the fragment sizes of CMS and its restorer line. The fragments of the size of 1.58 kb (Fig. 3, lane 7), and 1.38 kb (Fig. 3, lane 11) synthesized by OPS4 and OPS5, respectively appeared in the R line and remained absent in the A line. It was further observed that the primers OPC15, OPC16 and OPG13 synthesized unique fragments of 3.90 kb (Fig. 1, lane 1), 1.59 kb (Fig. 1, lane 1) and 1.02 kb size (Fig. 2, lane 13) only in the mtDNA of the CMS line. These results thus indicated that the mitochondrial gene(s) associated with CMS of the present pearl millet system may lie in one of these uniquely synthesized fragments in the mtDNA of a male sterile line.

Parentage analysis of nuclear genome has revealed Mendelian inheritance for RAPD products [11, 12], and the segregation studies showed the expected dominant nature of RAPD markers [7,13,14], Mitochondrial DNA bands inherited from one parent to all the members of an F_1 progeny have been explained by the homozygous dominant genotype of the parent harbouring these bands [15,16]. Given a female crossing partner as the origin of "aberrantly" inherited bands, a possible explanation would be that the fragments shared by the female parent and all the F_1 progenies originate from maternally inherited mtDNA. In the present investigation, an attempt has been made to examine the polymorphism in the mtDNAs of the pearl millet system.

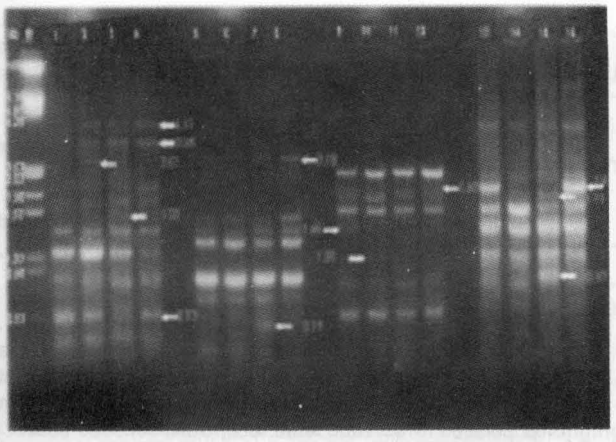

Fig. 1. RAPD analysis of pearl millet mt-DNA by random primers. Random primers: OPC-15 (lanes 1-4), OPC-16 (lanes 5-8), OPK-19 (lanes 9-12) and OPX-14 (lanes 13-16) primers. Lanes 1, 5, 9 and 13 - 1A; lanes 2, 6, 10 and 14 - 1B; lanes 3, 7, 11 and 15 - 138R; lanes 4, 8, 12 and 16-RHRBH8609, M - molecular weight marker DNA digested with *Eco*RI+*Hind*III

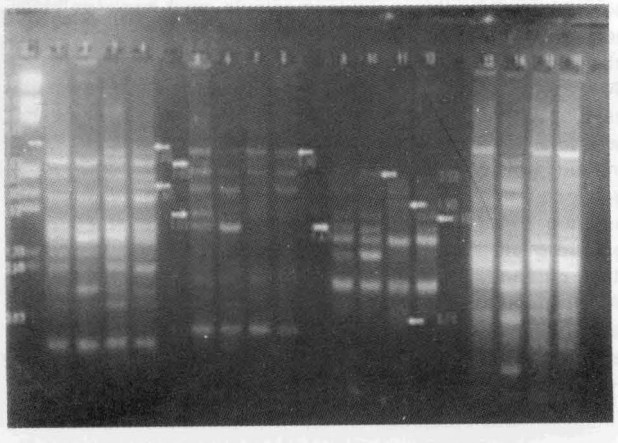

Fig. 2. RAPD analysis of pearl millet mt-DNA by random primers. Random primers: OPC-17 (lanes 1-4), OPC-15 (lanes 5-8), OPG-14 (lanes 9-12) and OPG-13 (lanes 13-16) primers. Lanes 1, 5, 9 and 13 - 1A; lanes 2, 6, 10 and 14 - 1B; lanes 3, 7, 11 and 15 - 138R; lanes 4, 8, 12 and 16-RHRBH8609, M - molecular weight marker DNA digested with *Eco*RI+*Hind*III

Lorenz *et al.* [17] observed differences in the banding patterns of mtDNA with five out of six primers in CMS and fertile lines of sugar beet. In the present investigation, 4 primers out of a total of 10 selected showed differences in the mtDNA of a male sterile and its isonuclear fertile line. This investigation thus confirms that mitochondrial genomes of the CMS system of pearl millet can be characterized and classified exclusively using some chosen RAPD markers. RFLP patterns provide useful information for detecting mtDNA diversity between different lines (5). However, a fairly large amount of purified mtDNA is required besides being time-consuming and probe radiolabelling.

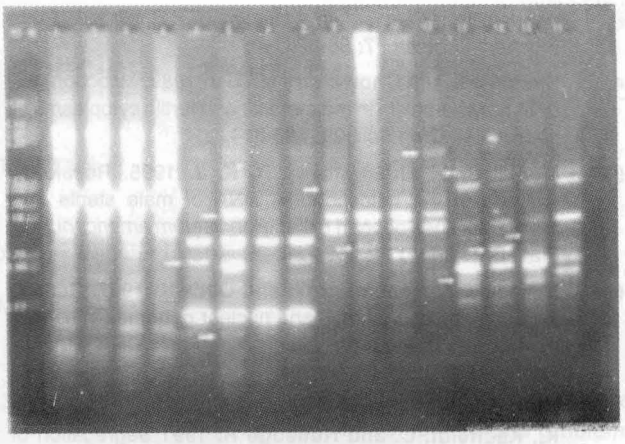

Fig. 3. RAPD analysis of pearl millet mt-DNA by random primers. Random primers: OPS-13 (lanes 1-4), OPS-4 (lanes 5-8), OPS-5 (lanes 9-12) and OPS-6 (lanes 13-16) primers. Lanes 1, 5, 9 and 13 - 1A; lanes 2, 6, 10 and 14 - 1B; lanes 3, 7, 11 and 15 - 138R; lanes 4, 8, 12 and 16-RHRBH8609, M - molecular weight marker DNA digested with *Eco*RI+*Hind*III

Grow-out test of the male-sterile lines to check the male sterility will be both laborious and time-consuming. In this context, the present investigation of mtDNA analysis by RAPD markers could be time-saving for detection of the possible contamination of male fertile plants in male-sterile plants in pearl millet.

In conclusion, the present investigation revealed four primers that distinguished between the mtDNA of A and B lines, whereas five primers detected differences in the A and R lines. Thus, this study will be useful to differentiate CMS line (having A_1 cytoplasm) from B and R lines. However, CMS systems having different cytoplasmic source needs to be screened by employing suitable RAPD primers.

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References

1. **Anand Kumar K. and Andrews D. J.** 1984. Cytoplasmic male sterility in pearl millet [*Pennisetum americanum* (L.) Leeke] : a review. Adv. Appl. Biol., **10**:113-143.
2. **Rai K. N. and Hash C. T.** 1990. Fertility restoration in male sterile and maintainer hybrids of pearl millet. Crop Sci., **30**: 889-892.
3. **Chhabra A. K., Rai K. N., Khairwal I. S., Sivaramakrishnan S. and Hash C. T.** 1998. Mitochondrial DNA-RFLP analysis distinguishes new CMS sources in

pearl millet [*Pennisetum glaucum* (L.) R. Br.] J. Plant Biochem. Biotech., **7**: 85-92.

4. **Smith R. L. and Chowdhury M. J. U.** 1989. Mitochondrial DNA polymorphism in male-sterile and fertile cytoplasms of pearl millet. Crop Sci., **29**: 809-814.
5. **Munjal S. V. and Narayan R. K. J.** 1995. Restriction analysis of the mitochondrial DNA of male sterile and maintainer lines of pearl millet, *Pennisetum americanum* L. Plant Breeding, **114**: 256-258.
6. **Nakajima Y., Yamamoto T. and Oeda K.** 1997. Genetic variation at mitochondrial and nuclear genomes in carrots revealed by random amplified polymorphic DNA (RAPD). Euphytica, **95**: 259-267.
7. **Carlson J. E., Tulsieram L. K., Glaubitz J. C., Luk V. W. K., Kauffeldt, C. and Rutledge R.** 1991 Segregation of random amplified DNA markers in F_1 progeny of conifers. Theor. Appl. Genet., **83**: 194-200.
8. **Chase C. D. and Pring D. R.** 1986. Properties of the linear N1 and N2 plasmid-like DNAs from mitochondria of cytoplasmic male- sterile *Sorghum bicolor*. Plant Mol. Biol., **6**: 53-64.
9. **Maniatis T., Fritsch B. and Sambrook J.** 1982. Molecular cloning : a laboratory manual : Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
10. **Samec P. and Nasinec V.** 1995. Detection of DNA polymorphism among pea cultivars using RAPD technique. Biol. Plant., **37**: 321-327.
11. **Welsh J., Honeycutt R. J., McClelland M. and Sobral B. W. S.** 1991. Parentage determination in maize hybrids using the arbitrarily-primed polymerase chain reaction (AP-PCR). Theor. Appl. Genet., **82**: 473-476.
12. **Waugh R. and Powell W.** 1992. Using RAPD markers for crop improvement. Trends Biotechnol., **10**: 186-191.
13. **Hu J. and Quiros C. F.** 1991. Identification of broccoli and cauliflower cultivars with RAPD markers. Plant Cell Rep., **10**: 505-511.
14. **Michelmore R. W., Paran I. and Kesseli R. V.** 1991. Identification of markers linked to disease-resistance genes by bulked segregation analysis : a rapid method to detect markers in specific genomic regions by using segregation population. Proc. Natl. Acad. Sci., USA. **88**: 9828-9832.
15. **Roy A., Frascaria N., MacKay J. and Bousquet J.** 1992. Segregating random amplified polymorphic DNAs (RAPDs) in *Betula alleghaniensis*. Theor. Appl. Genet., **85**: 173-180.
16. **Heun M. and Helentijaris T.** 1993. Inheritance of RAPDs in F_1 hybrids of corn. Theor. Appl. Genet., **85**: 961-968.
17. **Lorenz M., Weihe A. and Borner T.** 1994. DNA fragments of organellar origin in random amplified polymorphic DNA (RAPD) patterns of sugar beet (*Beta vulgaris* L.). Theor. Appl. Genet., **88**: 775-779.