A new source of cytoplasmic male sterility in pearl millet: RFLP analysis of mitochondrial DNA[1]

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A new source of cytoplasmic male sterility (cms) in pearl millet (*Pennisetum glaucum* (L.) R.Br.) derived from a half-sib progeny of the Early Gene Pool (EGP 261) and used in a male-sterile line, ICMA 90111, was compared with other known cms sources for RFLP of mitochondrial (mt) DNA. Southern blot hybridization of mtDNA from ICMA 90111 digested with several restriction enzymes and probed with homologous mtDNA clones from pearl millet and heterologous gene clones from maize and wheat revealed the RFLP patterns of ICMA 90111 distinct from others studied so far. The dendrogram of male-sterile lines constructed from the Southern blot hybridization patterns indicated that ICMA 90111 represents a separate group. Our results suggest that this source of cms is unique in several respects.

Key words: *Pennisetum glaucum*, cytoplasmic male sterility, mitochondrial DNA, RFLP.

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Une nouvelle source de stérilité mâle cytoplasmique (smc=cms) chez le millet perlé (*Pennisetum glaucum* (L.) R.Br.), dérivée d'une descendance semi-apparentée du Pool de Gènes Ancestraux 261 (Early Gene Pool = EGP 261), a été utilisée dans la lignée mâle-stérile ICMA 90111 et comparée avec d'autres sources connues de smc relativement aux polymorphismes des longueurs de fragments de restriction (PLFR) de l'ADN mitochondrial. Des hybridations de buvardages Southern de l'ADNmt de la lignée ICMA ont été soumises à la digestion de plusieurs enzymes de restriction, puis à l'activité de sondes de plusieurs clones d'ADNmt homologues provenant du millet perlé ainsi que de clones de gènes hétérologues provenant du maïs et du blé; les profils de PLFR de l'ICMA 90111 se sont révélés distincts des autres profils étudiés jusqu'ici. Un dendrogramme des lignées mâles-stériles, dressé à partir des profils des hybridations de buvardages Southern, a indiqué que la lignée ICMA représente un groupe séparé. Les résultats obtenus suggèrent que cette source de smc est unique sous plusieurs aspects.

Mots clés : *Pennisetum glaucum*, stérilité mâle cytoplasmique, ADM mitochondrial, PLFR.

[Traduit par la rédaction]

Introduction

Cytoplasmic diversification of male-sterile lines is an important aspect of any hybrid breeding program. In pearl millet (*Pennisetum glaucum* (L.) R. Br.), different sources of cytoplasmic male sterility (cms) have traditionally been classified by male fertility restoration patterns of their hybrids (Burton and Athwal 1967). Significant interaction of environmental factors and nuclear genetic background with the cytoplasm in the expression of fertility restoration of hybrids requires the use of isonuclear lines for the identification of cms sources (Schertz and Ritchey 1978; Rai and Hash 1990). This approach, however, is very time consuming. Cytoplasmic factors associated with male sterility have been shown to be encoded by the mitochondrial genome (Lonsdale 1987) and restriction fragment length polymorphism (RFLP) of mitochondrial DNA (mtDNA) has been shown to provide a rapid procedure to classify male-sterile cytoplasms (Pring et al. 1982; Bailey-Serres et al. 1986; Smith and Chowdhury 1989; Smith et al. 1987). Pring and Levings (1978) found that the maize cytoplasms S, C, T, and M had distinct mtDNA restriction patterns. Thirteen male-sterile lines of pearl millet from diverse cms sources were characterized by RFLP of mtDNA and shown to belong to four different groups (Rajeshwari et al. 1994). A new source of cms in pearl millet, derived from the Early Gene Pool (Rai and Hash 1993), was used to develop a male-sterile line ICMA 90111 at the ICRISAT Center. The objective of this study was to characterize the cytoplasm of ICMA 90111 by comparing the RFLP pattern of mtDNA from the other known cytoplasmic groups with that of ICMA 90111.

Materials and methods

Plant material

Five male-sterile lines (A lines) of pearl millet used in this study were ICMA 90111 and four near-isonuclear A lines, 81 A_1, ICMA 88001, 81 A_m, and Pb 406A_3. The 81 A_1 line, representative of A_1 cytoplasm, was derived from Tift 23 A_1 (Burton 1965) and Pb 406 A_3, representative of A_3 cytoplasm, was derived from the L67A_3 cms source (Burton and Athwal 1967). ICMA 88001 (A_v) was developed from *P. glaucum* subsp. *violaceum* (Lam.) L. Rich. (Marchais and Pernes 1985) and 81 A_m, representative of A_4 cytoplasm, was developed from *P. glaucum* subsp. *monodii* (= *violaceum*) (Maire) Brunken (Hanna 1987).

Clones used

A 4.7-kb *Pst*I fragment of pearl millet mtDNA was used as a probe and it was found only in the male-sterile mitochondria and contained the *cox*I and *rrn18–rrn5* gene fragments (Smith and

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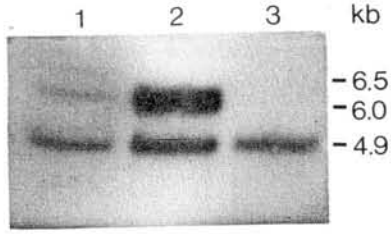

FIG. 1. Southern blot hybridization of mtDNA from different A lines of pearl millet digested with *Bam*HI and hybridized to a 4.7-kb pearl millet clone. Mitochondrial DNA was digested with *Bam*HI and separated on 0.8% agarose gel by electrophoresis. Lane 1, 81 A (representing the A_1 group of cytoplasm); lane 2, $81A_m$ (representing cytoplasms other than the A_1 group and ICMA 90111 and designated as the non-A_1 group of cytoplasm); and lane 3, ICMA 90111. Fragment sizes are indicated in kilobases.

Chowdhury 1991). This clone was provided by R.L. Smith, Department of Agronomy, University of Florida, Gainesville, Florida. Maize clone *atp6* (ATPase subunit 6, Dewey et al. 1985) was supplied by C.S. Levings III, Genetics Department, North Carolina State University, Raleigh, North Carolina. Maize clone *cox*I (cytochrome c oxidase subunit I, Isaac et al. 1985), was provided by C.J. Leaver, Department of Plant Science, University of Oxford, Oxford, U.K., and the wheat clone *cox*III (cytochrome c oxidase subunit III, Gualberto et al. 1990) was provided by J.M. Greinenberger, Institut de Biologie Moleculaire des Plantes, Strasbourg Cedex, France.

DNA analysis

Mitochondrial DNA was extracted from 5-day-old seedlings (about 50 g) grown in the dark, according to Smith et al. (1987). Mitochondrial DNA was digested with several restriction enzymes as per the supplier's instructions in the presence of RNase I (50 μg/mL) and the fragments were separated on 0.8% agarose gel using TAE (Tris–acetate 0.04 M, EDTA 0.001 M, pH 7.8) buffer. Southern transfer of DNA fragments onto a nylon membrane (Hybond N^+, Amersham) was performed using the capillary method. Blots were washed in 3× SSC (0.45 M NaCl, 0.045 M sodium citrate, pH 7.0) after transfer and UV cross-linked as described by Smith et al. (1987).

The random primed labelling method of Feinberg and Vogelstein (1983) was used for the preparation of ^{32}P-labelled probes. Southern blots were prehybridized with 30 mL of a hybridization solution containing 7% SDS, 1% BSA, 0.5 M Na_2HPO_4, pH 7.2, and 100 μg/mL sheared, denatured Salmon sperm DNA per two blots (20 × 15 cm size). Hybridizations were carried out by adding the labelled probe to the prehybridization solution and incubating for 16 h at 65°C in standard bottles (30 × 3.5 cm, l × d) in a hybridization oven (Hybaid, U.K.). The blots were washed three times in 3× SSC containing 0.1% SDS at 65°C for 30 min each. Autoradiography of the blots was conducted at −70°C for varying periods using X-AR (Kodak) film. The fragment size was determined by using standard DNA markers.

Similarity index matrices were generated based on the proportion of common restriction fragments between two lanes (Nei 1987) using

$$F = \frac{2M_{xy}}{M_x + M_y}$$

where F is the similarity index, M_x is the number of bands in accession x, M_y is the number of bands in accession y, and M_{xy} is the number of bands common to both x and y.

The genetic analysis was done based on the expression of similarity of objects and respective groups by the agglomeration method of hierarchical clustering techniques which proceed by a series of successive fusions of the "n" objects into clusters, using GENSTAT.

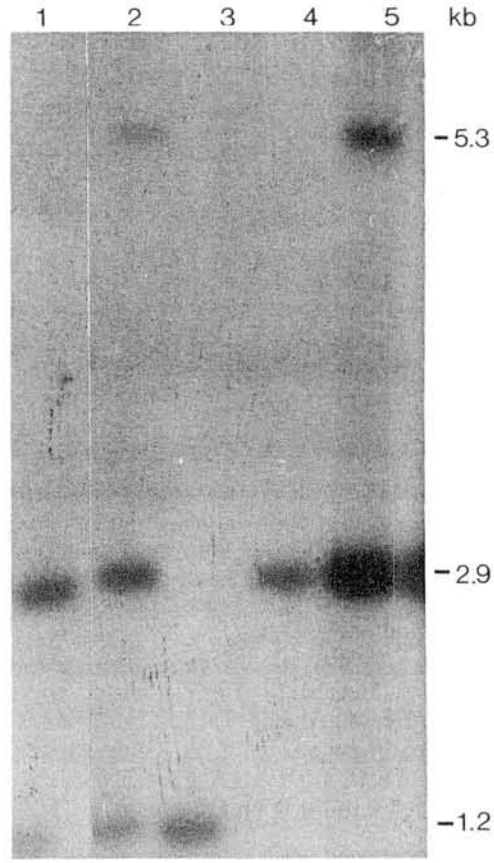

FIG. 2. Southern blot hybridization of mtDNA from different A lines of pearl millet digested with *Hin*dIII and probed with a maize gene *atp6* clone. Lane 1, 81 A; lane 2, Pb $406A_3$; lane 3, $81A_m$; lane 4, ICMA 88001 (A_v); lane 5, ICMA 90111. Fragment sizes are indicated in kilobases.

Results

The pearl millet 4.7-kb clone revealed three types of hybridization patterns with *Bam*HI-digested mtDNA. The A_1 group of cytoplasms represented by 81 A showed a characteristic hybridization pattern with two fragments 6.5 and 4.9 kb in size (Fig. 1, lane 1), whereas ICMA 90111 showed a single 4.9-kb fragment (Fig. 1, lane 3). All the other cytoplasms designated as the non-A_1 group of cytoplasms and represented by 81 A_m contained 6.5, 6.0, and 4.9 kb fragments (Fig. 1, lane 2). Southern blot hybridization patterns of *Bam*HI-digested mtDNA from 81 A, 81 A_m, and ICMA 90111 were identical to that obtained with the pearl millet 4.7-kb clone when probed with the maize *cox*I clone.

Two 5.3- and 2.9-kb *Hin*dIII fragments (Fig. 2, lane 5) hybridized to the maize *atp6* clone in ICMA 90111. Though the 2.9-kb fragment was shared by other A lines except 81 A_m, the 5.3-kb fragment was shared only by Pb $406A_3$ (a weakly hybridizing fragment). From the intensity of hybridization in ICMA 90111, it appears that there could be more gene copies of the 2.9-kb fragment than the 5.3-kb fragment. The mtDNA of ICMA 90111 digested with *Eco*RI and probed with the *atp6* clone revealed two fragments 6.6 and 4.9 kb in size (Fig. 3, lane 5) hybridizing with equal intensity. ICMA 88001 (Fig. 3, lane 4) shared the 6.6-kb

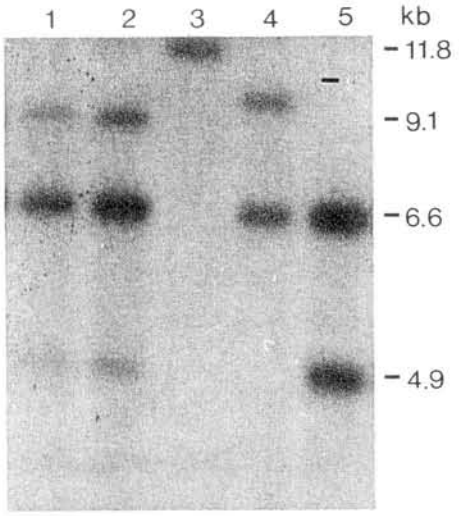

FIG. 3. Southern blot hybridization of mtDNA from different A lines of pearl millet digested with *Eco*RI and probed with a maize gene *atp6* clone. Lane 1, 81 A; lane 2, Pb 406A_3; lane 3, 81A_m; lane 4, ICMA 88001 (A_v); lane 5, ICMA 90111. Fragment sizes are indicated in kilobases.

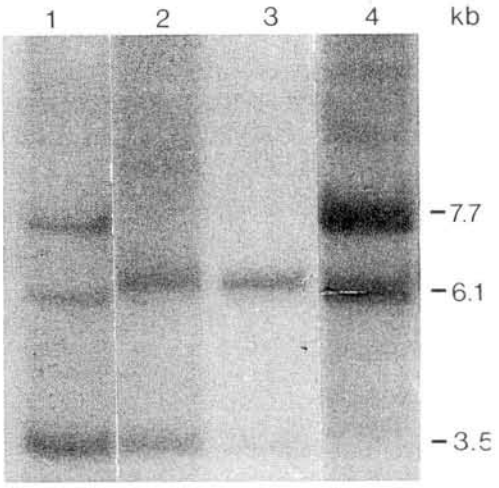

FIG. 4. Southern blot hybridization of mtDNA from different A lines of pearl millet digested with *Sma*I and probed with a wheat *cox*III gene clone. Lane 1, 81 A; lane 2, Pb 406A_3; lane 3, 81A_m; lane 4, ICMA 90111. Fragment sizes are indicated in kilobases.

fragment with ICMA 90111 (Fig. 3, lane 5) and the 9.1-kb fragment with Pb 406A_3 and 81 A (Fig. 3, lanes 1 and 2). The hybridization pattern of 81A_m showed only a single fragment 11.8 kb in size. 81 A gave a pattern similar to Pb 406A_3, though the 4.9-kb fragment in 81 A was very faint.

Southern blots of *Sma*I digests of mtDNA from four cytoplasms hybridized to the wheat gene probe *cox*III gave distinct hybridization patterns (Fig. 4). Two fragments 7.7 and 6.1 kb in size hybridizing with equal intensity were found in ICMA 90111 (Fig. 4, lane 4). Of these two fragments, the 6.1-kb fragment was present in all the cytoplasms and the 7.7-kb fragment was present only in 81 A (Fig. 4, lane 1) and ICMA 90111 (Fig. 4, lane 4). A 3.5-kb fragment present in 81 A (Fig. 4, lane 1) and Pb 406A_3 (Fig. 4, lane 2) was absent in 81A_m (Fig. 4, lane 3) and ICMA 90111 (Fig. 4, lane 4). ICMA 88001 gave a hybridization pattern distinct from the others, but the bands were very faint (data not included).

Cluster analysis was done on the data obtained from the Southern blot hybridization patterns for each enzyme–probe combination, as well as multiple enzyme–probe combinations. The dendrogram generated from the hybridization patterns of mtDNA digested with *Bam*HI, *Hin*dIII, *Pvu*II, *Eco*RV, and *Sma*I and probed with the maize *cox*I and the pearl millet 4.7-kb clone is shown in Fig. 5. ICMA 90111 formed a very distinct group at $F = 0.32$ (Fig. 5). The dendrogram based on similarity indices generated from the hybridization patterns of mtDNA digested with *Bam*HI, *Hin*dIII, *Pvu*II, *Eco*RI, and *Sma*I and probed with the maize *atp6* clone (Fig. 6) revealed more groups compared to *cox*I. ICMA 90111 formed a separate group from the others ($F = 0.45$) in a total of five groups.

Discussion

The homologous pearl millet clones and maize *cox*I are known to distinguish only two groups of cytoplasms, A_1 versus the non-A_1 group (consisting of all other cytoplasms identified so far and their B-lines), with many probe–enzyme combinations (Sivaramakrishnan et al. 1993). The maize *cox*I and pearl millet 4.7-kb clones identified ICMA 90111 as a distinct cms group. ICMA 90111 revealed a single hybridization fragment with the maize *cox*I and pearl millet 4.7-kb clone, while the A_1 and non-A_1 group (excluding ICMA 90111) revealed a greater number of fragments. The 4.7-kb clone corresponds to the *rrn18–rrn5/cox*I region of maize, which may be a reason for the similar hybridization patterns of the 4.7-kb pearl millet and maize *cox*I clones (Smith and Chowdhury 1991). The hybridization pattern of ICMA 90111 mtDNA digested with *Hin*dIII, *Sma*I, *Eco*RV, and *Eco*RI and probed with *cox*I revealed single fragments of 3.8, 6.7, 12.8, and 13.5 kb, respectively (data not shown). This could possibly be due to the presence of cleavage sites for the different restriction enzymes outside the *cox*I gene region. The unique hybridization pattern obtained with *cox*I and many enzyme combinations suggests that the *cox*I gene might have undergone some rearrangements in ICMA 90111. This probe could thus be used for the identification of ICMA 90111. This is also supported by the cluster analysis of hybridization pattern generated by the combination of different enzymes and *cox*I gene clone as probe, where ICMA 90111 formed an exclusive group at $F = 0.32$ (Fig. 5).

Cluster analysis of the data from Southern blot hybridization patterns with *atp6* and different restriction enzymes identified ICMA 90111 as a separate group (Fig. 6). Maize *atp6* clone is known to distinguish male-sterile cytoplasms into four major groups (Smith and Chowdhury 1989; Sivaramakrishnan et al. 1993). All the A_1 and A_3 lines formed two separate clusters, and ICMA 88001 (A_v) and 81A_m were found to differ from each other as well as from the others. Superimposition of the present data of ICMA 90111 on the dendrogram constructed by Sivaramakrishnan et al. (1993) formed the fifth group between ICMA 88001 (A_v) and 81A_m.

The wheat *cox*III gene probe revealed four distinct hybridization patterns for 81 A, Pb 406A_3, 81A_m, and ICMA 90111. The present data indicate that the *cox*III gene clone of wheat could also distinguish many of the pearl millet cytoplasmic systems, similar to the *atp6* gene probe of maize (Smith and Chowdhury 1989; Sivaramakrishnan et al. 1993). This also indicates the conservation of mtDNA

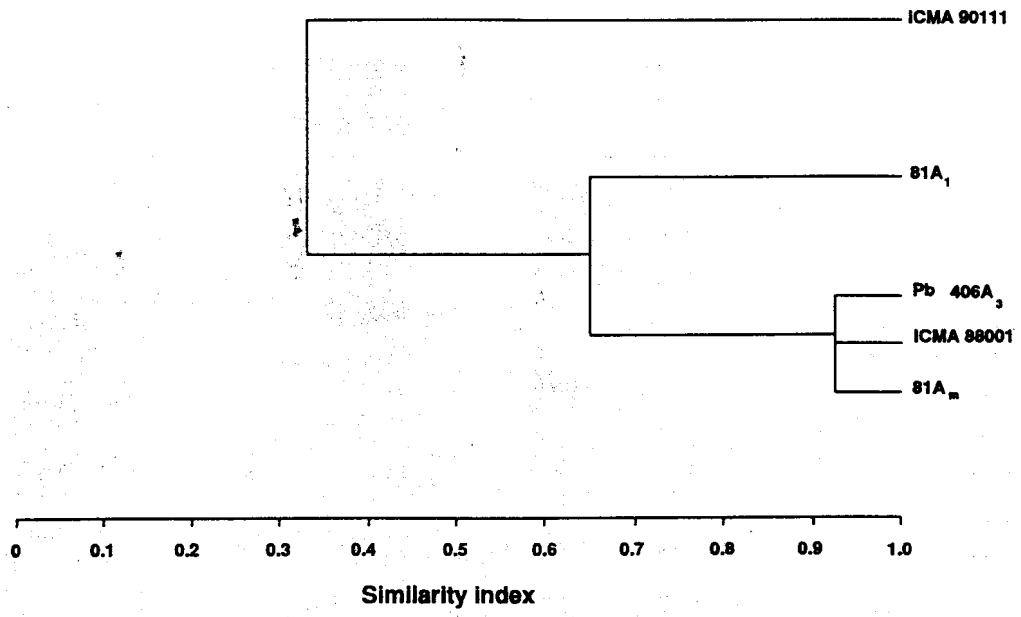

FIG. 5. Dendrogram of cytoplasmic male-sterile lines of pearl millet based on maize *cox*I and pearl millet 4.7-kb clones. Cluster analysis of the data obtained on the Southern blot hybridization of various male-sterile lines of pearl millet mtDNA digested with *Bam*HI, *Hin*dIII, *Eco*RI, *Pvu*II, *Sma*I, and *Eco*RV and hybridized to the maize *cox*I and pearl millet 4.7-kb clones was done as described in the Materials and methods.

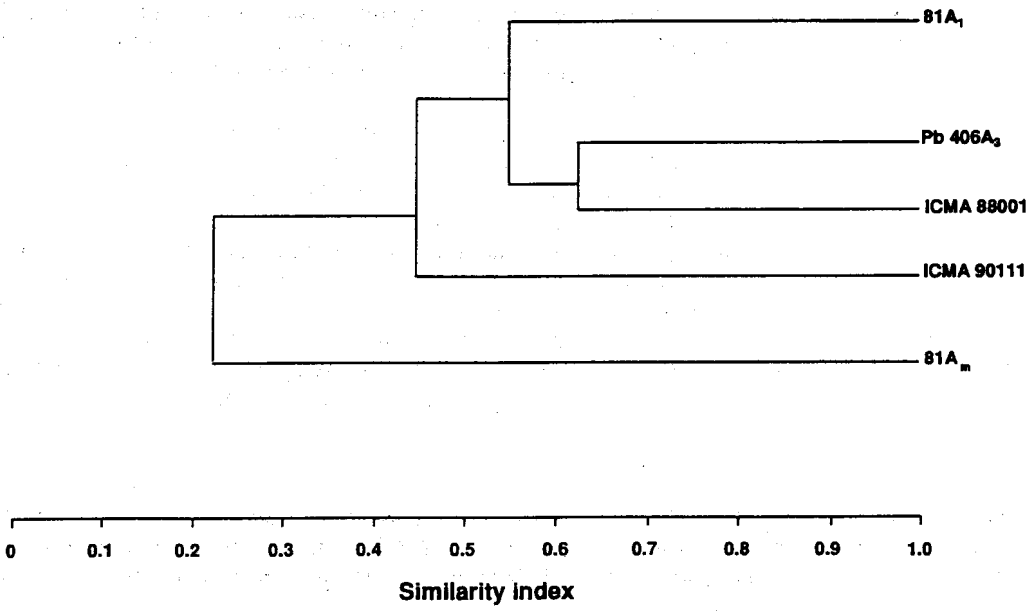

FIG. 6. Dendrogram of cytoplasmic male-sterile lines of pearl millet based on the maize *atp6* clone. Cluster analysis of the data obtained on the Southern blot hybridization of various male-sterile lines of pearl millet mtDNA digested with *Bam*HI, *Hin*dIII, *Eco*RI, and *Eco*RV and hybridized to the maize *atp6* clone was done as described in the Materials and methods.

sequences among the crop species as wheat and maize probes could detect the polymorphism in pearl millet.

The present study suggests that ICMA 90111 possibly contains a unique mitochondrial genome that is different from several others reported earlier (Sivaramakrishnan et al. 1993; Smith and Chowdhury 1989). The uniqueness of ICMA 90111 is revealed in the different Southern blot hybridization patterns with specific enzyme-probe combinations.

The mtDNA rearrangements resulting in the formation of chimeric genes have been implicated as one of the mechanisms of male sterility in many crop species (Leaver and Lonsdale 1988). Functional chimeric genes, such as the *pcf* gene in *Petunia* (Nivson and Hanson 1989), containing sequences from *atp9*, coxII, and an unidentified reading frame are expressed as proteins that are associated with cms. In rice, a chimeric gene with portions of the *atp6* gene is reported to be associated with cms (Kadowski et al. 1990). In a similar way, rearrangements involving *cox*I, *cox*III, and *atp6* could possibly be responsible for cms in pearl millet.

The $81A_m$ cytoplasm has been reported to be stable (Hanna 1989). However, a majority of the hybrids with the cytoplasm with restorers of the A_1 cytoplasm are male sterile (Hanna 1989; Rai and Hash 1993). The male-sterile line ICMA 90111 was found to be stable and can provide an alternative to the existing stable cms sources in diversifying the cytoplasmic base of male-sterile lines. Preliminary studies,

though not based on near-isonuclear A line, indicates that the frequency of male-sterile hybrids on ICMA 90111 is considerably more than on 81 A and substantially less than on $81A_m$ (Rai and Hash 1993). This would provide better opportunities to breed both male-sterile lines and restorers on ICMA 90111 cms source than was possible on either of the A_1 or A_m cms systems.

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