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Full Length Research Paper

Structural features of a cytoplasmic male sterility source from *Helianthus resinosus,* CMS RES1

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Comparisons in the structural organization of mitochondrial DNA between a cytoplasmic male sterility source from *Helianthus resinosus* (CMS RES1) and HA89 fertile line have been performed surrounding *cox*III and *atp*9 genes. Differences have also been recognized by PCR analyses between HA89 (CMS RES1) and either HA89 (CMS PET1) or HA89 (CMS MAX1), indicating that the molecular basis of their sterility mechanism is different. To be specific, RES1 lacks *orf*H*522*, which is present in the mitochondrial DNA of PET1 and MAX1.

Key words: *Helianthus annuus*, *Helianthus resinosus*, sunflower, cytoplasmic male sterility, CMS, *cox*III, *atp*9.

INTRODUCTION

Hybrid breeding in sunflower is based on a single source of cytoplasmic male sterility (CMS), the so-called PET1, which was originated from an interspecific cross between *Helianthus petiolaris* and *H. annuus* (Leclercq, 1969). However, the use of a single CMS source implies a potential risk as a result of the vulnerability of such a narrow genetic basis (Haevekes et al., 1991). In sunflower, up to date 72 CMS sources are available and could be potentially used for hybrid breeding (Serieys and Christov, 2005). However, most of them lack detailed information about structural and functional characteristics. Therefore, thorough investigations on the molecular level of the available CMS are advisable to identify cytoplasms different in respect to the male sterility mechanism and the organization of the mitochondrial DNA (mtDNA).

Mapping studies of mtDNAs from isonuclear fertile and CMS PET1 lines have revealed that detectable alterations are restricted to an mtDNA region of about 16 kb, between *atp*A and *cob* genes. This region includes an 11 kb inversion and a 5 kb insertion, creating an open reading frame (*orf*H522) immediately downstream of the *atp*A gene (Sicullela and Palmer, 1988). Nine additional CMS sources also have the same organization of the *atp*A and CMS mechanism as PET1 (Horn and Friedt, 1999).

We have agronomically described a CMS source from *H. resinosus,* CMS RES1. This cytoplasm is the result of controlled crosses between the perennial species *H. resinosus* (2n = 6x = 102) and cultivated sunflower *H. annuus* (2n = 2x = 34) followed by several backcrosses using *H. annuus* as a recurrent parent, in order to restore the diploid level. This cytoplasm is stable in different environments, the female-fertility is not affected and meiosis is normal. The absence of pollen is caused by postmeiotic alterations and the factors which control the pollen fertility restoration have not been found in diploid germplasm (Echeverria et al., 2003).

In this communication, we report structural features from its mtDNA that show architectural differences in the region around *atp*6 – *cox*III genes from some fertile inbred lines, such as HA89, RHA271, and RHA801 compared with male – sterile lines, CMS RES1, as well as with HA89 (CMS PET1) and (CMS MAX1).

MATERIALS AND METHODS

Plant materials

CMS sources

The fertile inbred lines HA89 (B), RHA271 (B), RHA801 (B), and the male-sterile isoplasmic lines: HA89 (CMS MAX1), HA89 (CMS

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PET1), HA89 (CMS RES1), RHA271 (CMSRES1), RHA801 (CMSRES1) have been used. The inbred lines RHA271 (B) and RHA 801 (B) carry the CMS PET1 and the gene of pollen fertility restoration for this cytoplasm.

DNA isolation

Total DNA (tDNA) was obtained from leaves as indicated by Saghai-Maroof et al. (1984).

PCR analyses

Universal mitochondrial primers

Primers *cox*III5´/*cox*III3´ were used for the amplification of *cox*II gene (Iglesias, 1994). For amplifying the region *rp*S14 – *cob,* primers *rp*S14 and *cob* were used and for *nad* gene the pairs *nad4*exon1/*nad4*exon2 and *nad*4exon2/*nad*4exon4, were used according to Demesure et al. (1995).

Amplicons obtained by the utilization of primer pairs *cox*III *5'/3'*, *rp*S14/*cob* and *nad*4 exon2/4 were digested by using *Hae*III and *Hpa*II (Promega) in order to search for structural differences between lines.

Specific primers

Primers *orf*H*522F*/*orf*H*522R* for amplifying the sunflower specific region, were used according to Rambaud et al. (1997). A three-primer strategy, using primers *atpAF, orf*H*522R* and *orf*H*873R* homologous to portions of mitochondrial *atp*A, *orf*H522 and *orf*H*87*3, respectively, was applied as described by Rieseberg et al. (1994).

Southern hybridization

Mitochondrial probes

Probes for *atp*A (700 bp), *cox*II (1.1 kb) and *atp*9 (2.2 kb) were obtained by PCR amplification using pUC/M13 primers (Promega) and pUC plasmids containing the genes *atp*A (Laver et al., 1991), *cox*II and *atp*9 (Dewey et al., 1985). The probe for *orf*H522 was obtained by PCR amplification from HA89 (CMS PET1) total DNA (tDNA) using sequence specific primers *orf*H*522F/orf*H*522R* (Rambaud et al., 1997). All the PCR-amplified fragments were labeled with alkaline phosphatase using the AlkPhos Direct Labelling system (Amersham).

Total DNA was digested using the restriction enzymes *Bgl*II*, Eco*RI*, Hin*dIII *and Sal*I (Promega), according to the manufacturer's manuals.

Southern blot

Digested tDNAs (2µg) were separated on a 1% agarose gel and blotted onto positively charged nylon membranes (Hybond-N+, Amersham). Hybridization was performed according to Sambrook et al. (1989). After hybridization, the specific fragments were detected by chemiluminescence using the AlkPhos Detecting system (Amersham).

RESULTS

PCR analyses

Universal mitochondrial primers

No differences in amplified fragment sizes were observed between HA89 fertile, CMS PET1, and CMS RES1, from the genes *cox*III (700 bp product), *rp*S14-*cob* (1000 bp product)*,* and *nad4* (2 kb for the stretch between exon 1 and 2, and 600 bp from exon 2 to 4). Restriction analyses, using *Hae*III and *Hpa*II on PCR products obtained by using *cox*III, *nad*4 (exon 2/4) and *rpS14/cob* primer pairs, did not detect any difference among the amplified products from HA89 fertile, CMS PET1 and CMS RES1 lines (not shown).

Specific primers

When PCR analyses were performed using *orf*H522 primer pairs, no amplification was detected when tDNA of HA89 (B) was used as template, and fragments of 344 bp were observed using tDNA from HA89 (CMS PET1) and HA89 (CMS MAX1), as expected. When the same analysis was carried out using tDNA from HA89 (CMS RES1) no amplification was obtained**.** These results were confirmed using tDNA from the inbred lines RHA271and RHA801 fertile lines and the isoplasmic form (CMS RES1) (data not shown). These fertile lines show the fragment of 344 bp, because they carry the CMS PET1. Similarly, when using the three-primer strategy (Figure 1A), the expected fragments of 1450, 840 and 790 bp, were obtained when the technique was applied on HA89 (CMS PET1), HA89 (B) and HA89 (CMS MAX1) tDNAs, respectively (Figure 1A, Lanes 1, 2 and 4) (Rieseberg et al., 1994; Hahn and Friedt, 1994 and GenBank Acc# X55963). The fertile inbred lines RHA271 and RHA801 exhibited the fragment of 1450 bp, (Figure 1B, Lanes 2 and 3) like CMS PET1. As on the fertile line HA89 (B), (Figure 1A, Lane 2) fragments of 840 bp were amplified from HA89 (CMS RES1) (Figure 1A, Lane 3), RHA271 (CMSRES1) and RHA801 (CMSRES1) tDNA (Figure 1B, Lanes 5 and 6).

Southern hybridization

Southern blot analyses

Hybridization with *orf*H522 and *atp*A probes on tDNA samples digested with *Bgl*II, *Sal*I, *Hin*dIII or *Eco*RI showed no differences between fertile and CMS (RES1) lines (not shown). Similarly, the same hybridization pattern was observed on these lines when digested with *Hin*dIII and by using *cox*III and *atp*9 probes (not shown). However, we could detect structural differences by using *cox*III probe when both materials were digested with *Bgl*II or *Hin*dIII and by using *atp*9 probe on *Eco*RI digested tDNAs. Figure 2A shows that when using *cox*III probe on a *Bgl*II-digested DNA, a band of 8 kb was detected in the fertile line HA89 (Figure 2A, Lane 1) while a 5.5 kb band hybridized in the CMS RES1 (Figure 2A, Lane 2). In addition, the same analysis on

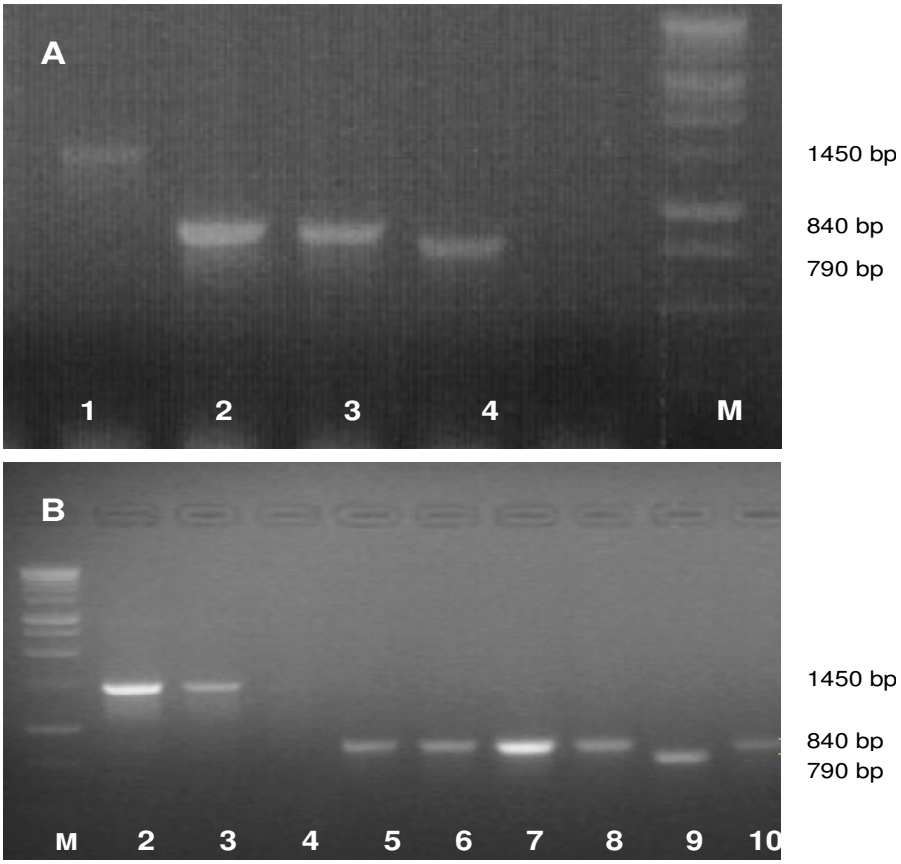

Figure 1. PCR analyses using the three-primer strategy. A: on tDNA from HA89 CMS PET1 (1), HA89 (B) (2), HA89 CMS RES1 (3), and HA89 CMS MAX1 (4). M: 1 Kb DNA ladder. B: on tDNA from RHA271 fertile (B) (2) and male-sterile (CMSRES1) (5); RHA801 fertile (B) (3) and male-sterile (CMSRES1) (6); and HA89 fertile (B) (7) and male-sterile isoplasmic lines (CMS RES1 (8), MAX1 (9) and PET2 (10). M: 1 Kb DNA ladder.

*Hin*dIII-digested tDNAs, showed a band of 7 kb in the fertile line HA 89 (Figure 2A, Lane 3) but exhibited a 5 kb fragment in the case of CMS RES1 (Figure 2A, Lane 4). Finally, when a fragment corresponding to *atp*9 gene was employed as probe on an *Eco R*I-digested tDNAs, the results were that, while a 6 kb fragment was recognized in both lines (Figure 2B, Lanes 5 and 6), a fragment of 18.5 kb detected in HA89 (B) (Figure 2B, Lane 5) was not present in CMS RES1 and an 11 kb fragment was hybridized on this material (Figure 2B, Lane 6).

DISCUSSION

Despite their apparent unrelated nature, most CMS associated mitochondrial genes share some characteristics. A common feature of CMS-associated genes is their physical association and co-transcription with essential mitochondrial genes. CMS genes appear as the products of the recombination activity of mitochondrial genomes in plants. Investigations in a variety of plant species including maize, *Petunia*, sunflower, *Brassica* and bean have shown that different alterations of the mitochondrial genome are associated with the CMS phenotype (Budar and Pelletier, 2001). A hypothesis suggests that CMS gene products somehow interfere with the normal physiology of mitochondria (possibly via the portions of normal mitochondrial polypeptides included in their sequences), leading to less efficient respiration and/or ATP production, impairing pollen production, which is an energy demanding developmental program (Budar et al., 2003).

Southern blot analyses showed that several CMS sources of the genus *Helianthus* presented differences in the hybridization patterns of the regions of *cox* and *atp* gene families (Horn, 2002). Structural alterations around these genes are also present in the CMS lines in other species like *Petunia* (Young and Hanson, 1987), *Brassica napus* (Dieterich et al., 2003), carrot (Szklarczyk et al., 2000), bean (Chase, 1994), *Lolium perenne* (Kiang and Kavanagh, 1996), and rice (Eckardt, 2006). Schnable and Wise (1998) reported differences between the male-sterile and male-fertile mitochondrial genomes that are confined to a rearranged region around the *atp6, atp9,* and *atpA*

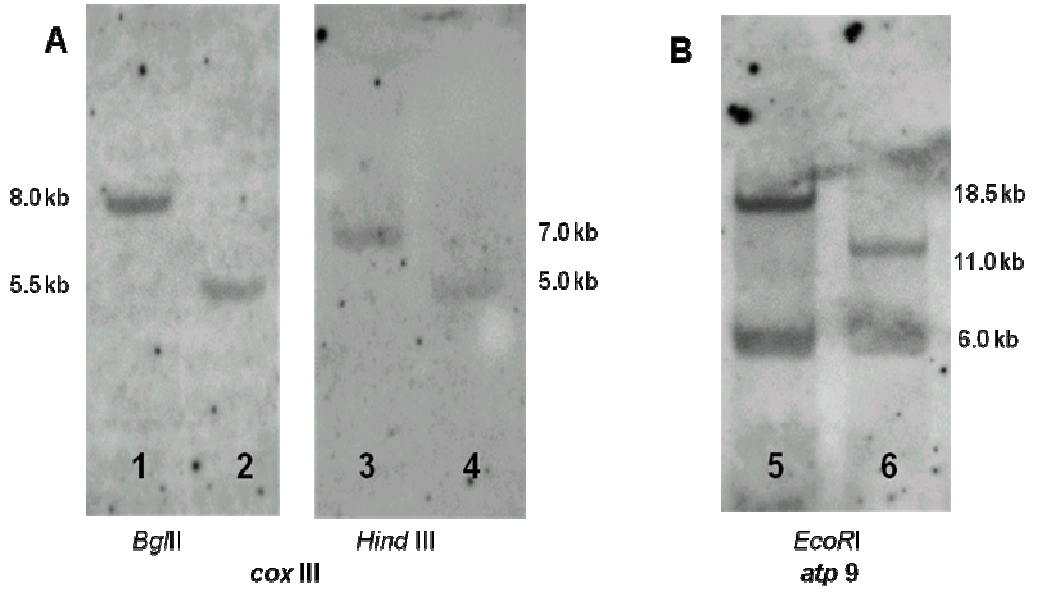

Figure 2. Southern blot hybridization. A: The probe was a fragment from *cox*III gene. tDNA from HA89 (B) (1, and 3) and from HA89 CMS RES1 (2, and 4), were digested with *Bgl*II (1, and 2) or *Hin*dIII (3, and 4). **B:** The probe was a fragment of *atp9* gene. tDNA from HA89 (B) (5) and from HA89 CMS RES1 (6) were digested with *Eco*RI.

genes. Specifically, the central role of the abnormal transcripts of *atp*9 linked to male sterility was reported in *Brassica napus* (Dieterich et al., 2003), *Petunia* (Hanson et al., 1996), and sorghum (Tang et al., 1996). In sunflower, Spassova et al. (1994) detected this *locus* in the cytotype CMS3, then De la Canal et al. (2001) explained the male sterility condition in CMS PEF1 by the insertion of 500 bp in the 3´*atp*9 gene.

We have shown here some structural features of CMS RES1 mtDNA. Amplicon sizes obtained through PCR amplification using primers *orf*H*522F* and *orf*H*522R* on CMS PET1 and CMS MAX1 tDNA were the same as those of Rambaud et al. (1997). When the same analytical strategy was applied on CMS RES1 no amplification was observed. These results indicate that *orf*H*522* is not involved in the male sterility condition in RES1 and that CMS RES1 has a different architectural organization than CMS PET1 and MAX1. When using the three primers system, the results pointed out to structural similarities between HA89 (B) and the three lines CMS RES1 in the *3´atp*A - *orf*H*873* region and, importantly, confirmed the differences that CMS RES1 has, in this region, with CMS PET1 and CMS MAX1 (Figure 1). In addition, CMS RES1 showed no differences associated with *rp*S14, *cob,* and *nad4* regions with the fertile line by PCR criteria.

On the other hand, Southern blot analyses showed that CMS RES1 also presents some differences in the mtDNA architecture compared to the fertile line. We detected hybridization differences between fertile HA89 (B) and CMS RES1 lines in areas surrounding *atp*9 and *cox*III genes (Figure 2). Therefore, these areas could locate structural differences responsible for the sterile phenotype RES1.

Studies are underway to further describe the molecular nature of the CMS RES1 and determine the sequences of mtDNA in the areas surrounding the *loci atp*9 and *cox*III to assess the homologies and differences in these areas with other CMS sources.

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REFERENCES

Budar F, Pelletier G (2001). Male sterility in plants: occurrence, determinism, significance and use. C. R. Acad. Sci. III 324: 543-550.

Budar F, Touzet P, De Paepe R (2003). The nucleo-mitochondrial conflict in cytoplasmic male sterilities revisited. Genetica 117: 3-16.

Chase CD (1994). Expression of CMS-unique and flanking mitochondrial DNA sequences in *Phaseolus vulgaris* L. Curr. Genet. 25: 245-251.

Demesure B, Sodzi N, Petit RJ (1995). A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. Mole. Ecol. 4: 192-131.

De la Canal L, Crouzillat D, Quetier F, Ledoigt G (2001). A transcriptional alteration on the atp9 gene is associated with a sunflower male-sterile cytoplasm. Theor. Appl. Genet. 102: 1185-1189.

Dewey R, Schuster A, Levings C, Timothy D (1985). Nucleotide sequence of F_0-ATPase proteolipid (subunit 9) gene of maize mitochondria. Proc. Natl. Acad. Sci. USA, 82: 1015-1019.

Dieterich J, Braun H, Schmitz U (2003). Alloplasmic male sterility in *Brassica napus* (CMS "Tournefortii-Stiewe") is associated with a special gene arrangement around a novel atp9 gene. Mole.. Genet. Genomics. 269: 723-731.

Eckardt NA (2006). Cytoplasmic male sterility and fertility restoration. Plant Cell 18, 515-517.

Echeverria MM, Salaberry MT, Rodríguez RH (2003). Characterization for agronomic use of cytoplasmic male-sterility in sunflower (*Helianthus annuus* L.) introduced from *H. resinosus* Small. Plant Breed, 122: 357-361.

Haevekes FWJ, Miller JF, Jan CC (1991). Diversity among sources of cytoplasmic male sterility in sunflower (*Helianthus annuus* L.). Euphytica, 5: 125-129.

Hahn V, Friedt W (1994). Molecular analysis of the cms-inducing MAX1 cytoplasm in sunflower. Theor. Appl. Genet. 89: 379-385.

Hanson MR, Sutton CA, Lu B (1996). Plant organelle gene expression: altered by RNA editing. Trends Plant Sci. 1: 57-64.

Horn R (2002). Molecular diversity of male sterilty inducing and male-fertile cytoplasms in the genus *Helianthus*. Theor. Appl. Genet. 104: 562-570.

Horn R, Friedt W (1999). CMS sources in sunflower: different origin but same mechanism? Theor. Appl. Genet. 98: 195-201.

Iglesias V (1994). Genetic transformation studies in wheat using particle bombardment. Swiss Federal Institute of Technology, Zurich. Dis # 10628.

Kiang A, Kavanagh T (1996). Cytoplasmic male sterility (CMS) in *Lolium perenne* L, 2. The mitochondrial genome of a CMS line is rearranged and contains a chimaeric atp9 gene. Theor. Appl. Genet. 92: 308-315.

Laver H, Reynolds S, Moneger F, Leaver C (1991). Mitochondrial genome organization and expression associated with cytoplasmic male sterility in sunflower (Helianthus annuus). Plant J. 1: 185-193.

Leclercq P (1969). Une stérilité mâle cytoplasmique chez le tournesol. Ann. Amélior. Plantes 19: 99-106.

Rambaud C, Bellamy A, Dubreucq A, Bourquin J, Vasseur J (1997). Molecular analysis of the fourth progeny of plants derived from a cytoplasmic male sterile chicory cybrid. Plant Breed. 116: 481-486.

Rieseberg LH, Van Fossen C, Arias D, Carter RL (1994). Cytoplasmic male sterility in sunflower: Origin, inheritance, and frequency in natural populations. J. Heredity 85: 233-238.

Saghai-Maroof M, Soliman K, Jorgensen R, Allard R (1984). Ribosomal DNA spacer length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc. Natl. Acad. Sci. USA 78: 7463-7467.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular cloning: A laboratory manual. 2nd ed. Vol 2. Cold spring Harbor, New York, USA, pp. 9.31-9.57.

Schnable PS, Wise RP (1998). The molecular basis of cytoplasmic male sterility and male fertility restoration. Trends Plant Sci. 3: 175-180.

Serieys H, Christov M (2005). Progress Report 1999-2004 activities of the FAO working group: Identification, study and utilization in breeding programs of new CMS sources. European Cooperative Research Network on Sunflower. Proc. X FAO Consultation Meeting, Novi Sad, Serbia and Montenegro, July 17-20, 2005. FAO, Rome, Italy.

Sicullela L, Palmer J (1988). Physical and gene organization of mitochondrial DNA in fertile and male sterile sunflower, CMS-associated alterations in structure and transcription of the ATP A gene. Nucl. Acids Res. 16: 3787-3799.

Spassova M, Moneger F, Leaver CJ, Petrov P, Atanassov A, Nijkamp P (1994). Characterization and expression of the mitochondrial genome of a new type of cytoplasmic male-sterile sunflower. Plant Molec. Bio. 26: 1819-1831.

Szklarczyk M, Oczkowski M, August VH, Borner T, Linke B, Michalik B (2000). Organization and expression of mitochondrial atp9 genes from CMS and fertile carrots. Theor. Appl. Genet. 100: 263-270.

Tang HD, Pring DR, Shaw LC, Salazar RA, Muza FR, Yan B, Schertz KF (1996). Transcript processing internal to a mitochondrial open reading frame is correlated with fertility restoration in male-sterile sorghum. Plant J. 10: 123-133.

Young E, Hanson M (1987). A fused mitochondrial gene associated with cytoplasmic male sterility is developmentally regulated. Cell 50: 41-49.