

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 *coxIII* and *atp9* 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 *orfH522*, which is present in the mitochondrial DNA of PET1 and MAX1.

Key words: *Helianthus annuus*, *Helianthus resinosus*, sunflower, cytoplasmic male sterility, CMS, *coxIII*, *atp9*.

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 cytoplasmic 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 *atpA* and *cob* genes. This region includes an 11 kb inversion and a 5 kb insertion, creating an open reading frame (*orfH522*) immediately downstream of the *atpA* gene (Sicullela and Palmer, 1988). Nine additional CMS sources also have the

same organization of the *atpA* 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 *atp6* – *coxIII* 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-Marouf et al. (1984).

PCR analyses

Universal mitochondrial primers

Primers *coxIII5'*/*coxIII3'* were used for the amplification of *coxIII* gene (Iglesias, 1994). For amplifying the region *rpS14 – cob*, primers *rpS14* and *cob* were used and for *nad* gene the pairs *nad4exon1/nad4exon2* and *nad4exon2/nad4exon4*, were used according to Demesure et al. (1995).

Amplicons obtained by the utilization of primer pairs *coxIII 5'3'*, *rpS14/cob* and *nad4 exon2/4* were digested by using *HaeIII* and *HpaII* (Promega) in order to search for structural differences between lines.

Specific primers

Primers *orfH522F/orfH522R* for amplifying the sunflower specific region, were used according to Rambaud et al. (1997). A three-primer strategy, using primers *atpAF*, *orfH522R* and *orfH873R* homologous to portions of mitochondrial *atpA*, *orfH522* and *orfH873*, respectively, was applied as described by Rieseberg et al. (1994).

Southern hybridization

Mitochondrial probes

Probes for *atpA* (700 bp), *coxIII* (1.1 kb) and *atp9* (2.2 kb) were obtained by PCR amplification using pUC/M13 primers (Promega) and pUC plasmids containing the genes *atpA* (Laver et al., 1991), *coxIII* and *atp9* (Dewey et al., 1985). The probe for *orfH522* was obtained by PCR amplification from HA89 (CMS PET1) total DNA (tDNA) using sequence specific primers *orfH522F/orfH522R* (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 *BglII*, *EcoRI*, *HindIII* and *SalI* (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 *coxIII* (700 bp product), *rpS14-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 *HaeIII* and *HpaII* on PCR products obtained by using *coxIII*, *nad4* (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 *orfH522* 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 RHA271 and 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 *orfH522* and *atpA* probes on tDNA samples digested with *BglII*, *SalI*, *HindIII* or *EcoRI* showed no differences between fertile and CMS (RES1) lines (not shown). Similarly, the same hybridization pattern was observed on these lines when digested with *HindIII* and by using *coxIII* and *atp9* probes (not shown). However, we could detect structural differences by using *coxIII* probe when both materials were digested with *BglII* or *HindIII* and by using *atp9* probe on *EcoRI* digested tDNAs. Figure 2A shows that when using *coxIII* probe on a *BglII*-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

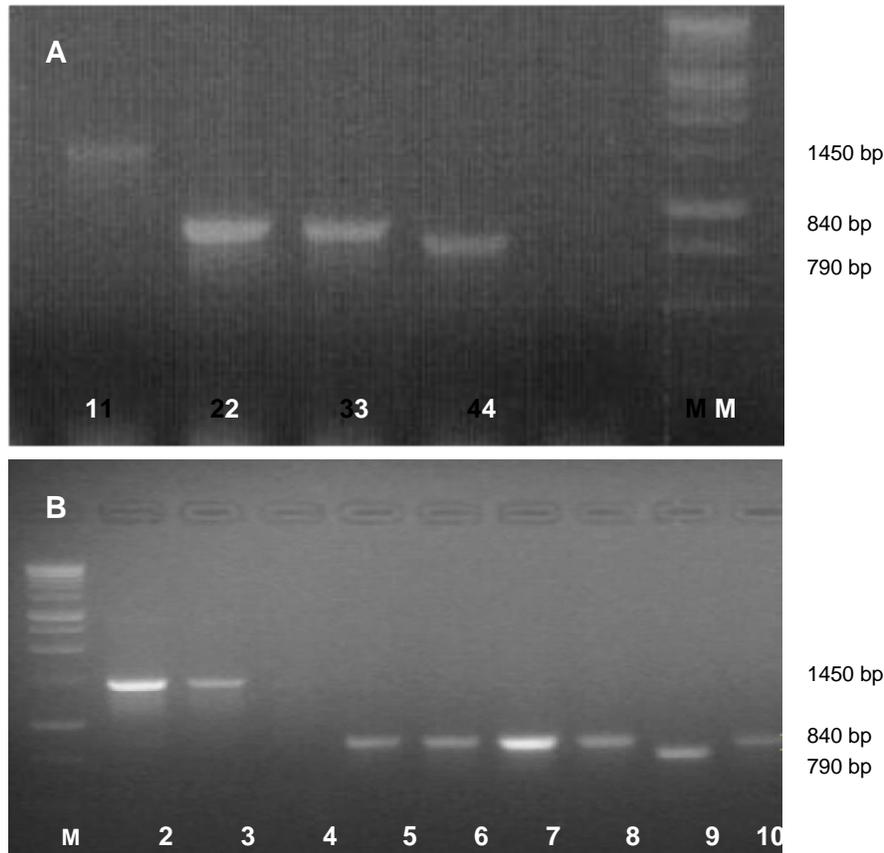


Figure 1. PCR analyses using the three-primer strategy. A: on tDNA from HA89 CMS PET1 (1), HA89 (B) (2), HA89 CMS RES1 (3), and HA89 CMS MAX1 (4). M: 1 Kb DNA ladder. B: on tDNA from RHA271 fertile (B) (2) and male-sterile (CMSRES1) (5); RHA801 fertile (B) (3) and male-sterile (CMSRES1) (6); and HA89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

*Hind*III-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 *atp9* gene was employed as probe on an *Eco* RI-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*

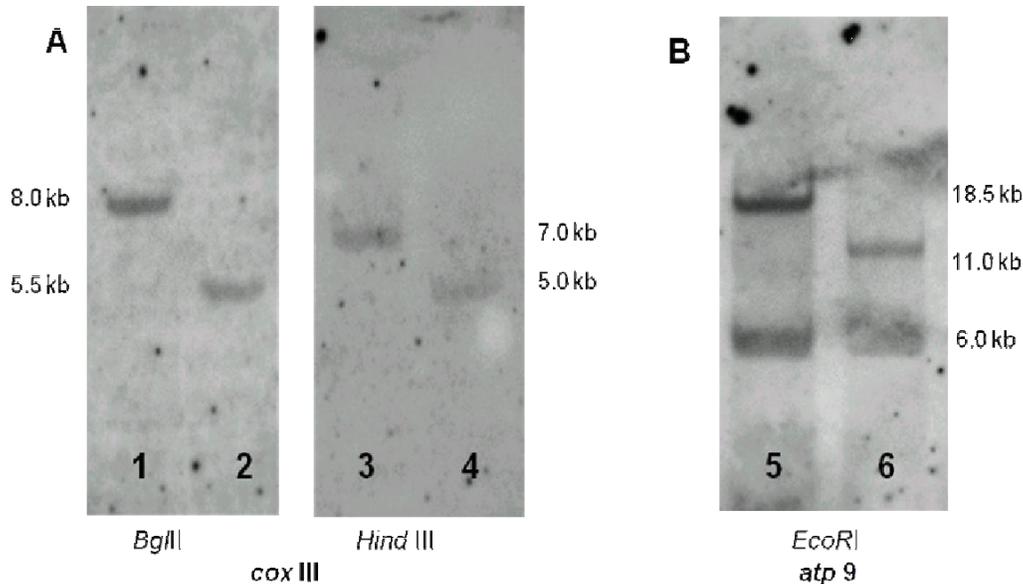


Figure 2. Southern blot hybridization. A: The probe was a fragment from *coxIII* gene. tDNA from HA89 (B) (1, and 3) and from HA89 CMS RES1 (2, and 4), were digested with *Bgl*II (1, and 2) or *Hind*III (3, and 4). B: The probe was a fragment of *atp9* gene. tDNA from HA89 (B) (5) and from HA89 CMS RES1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

genes. Specifically, the central role of the abnormal transcripts of *atp9* 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' *atp9* gene.

We have shown here some structural features of CMS RES1 mtDNA. Amplicon sizes obtained through PCR amplification using primers *orfH522F* and *orfH522R* 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 *orfH522* is not involved in the male sterility condition in RES1 and that CMS RES1 has a different architectural organization than CMS PET1 and CMS 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' *atpA* - *orfH873* 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 *rpS14*, *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 *atp9* and *coxIII* 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 atp9* and *coxIII* to assess the homologies and differences in these areas with other CMS sources.

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