Molecular analysis of a new cytoplasmic male sterile genotype in sunflower

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Mitochondrial DNA from 1 fertile and 6 cytoplasmic male sterile (CMS) sunflower genotypes was studied. The CMS genotypes had been obtained either by specific crosses between different Helianthus species or by mutagenesis. CMS-associated restriction fragment length polymorphisms (RFLPs) were found in the vicinity of the attA locus, generated by various restriction enzymes. The organization of the mitochondrial genes 26S rRNA, 18S+5S rRNA and coxII was investigated by Southern blot analysis. These genes have similar structures in fertile and all studied sterile sources. Using the attA probe, 5 from the 6 investigated CMS genotypes showed identical hybridization patterns to the Petunia CMS line which is used in all commercial sunflower hybrids. Only 1 cytoplasm derived from an open pollination of Helianthus annuus ssp. tschonoskei, known as ANT¹, contained a unique mitochondrial DNA fragment, which is distinguishable from the fertile and sterile Petunia CMS genotypes and from all investigated CMS genotypes. Male fertility restoration and male sterility maintenance of the ANT¹ line are different from the Petunia CMS system, which is a confirmation that a novel CMS genotype in sunflower has been identified.

1. INTRODUCTION

Cytoplasmic male sterility (CMS) is a maternally inherited trait in higher plants that results in the inability of the mature plant to produce functional pollen, but it does not affect female fertility [1]. In sunflower a CMS genotype was obtained from an interspecific cross between Helianthus petiolaris and H. annuus which was first described by Leclercq [2]. The subsequent identification of male fertile lines containing specific dominant nuclear genes which restore pollen fertility [3–5] resulted in a rapid production and cultivation of sunflower hybrids.

In a number of cases analysed, the CMS phenotype is suggested to originate from mutations in the mitochondrial genome of the male fertile progenitors as a result of intra- or intermolecular recombination events. The mitochondrial genome rearrangements have generated chimaeric mtDNA sequences which in some cases result in generation of novel mitochondrial genes or lead to a modification of existing genes [6]. These chimaeric genes are expressed as novel or modified polypeptides which, in an unknown fashion, are related to a failure in mitochondrial function during development of the pollen.

Our current knowledge about the molecular basis of CMS mainly comes from studies performed in maize and petunia. The chimaeric mitochondrial gene T-urf 13, composed primarily of sequences derived from the 26S rRNA, 18S+5S rRNA and atp6 gene, was investigated by Southern blot analysis. These genes have similar structures in fertile and all studied sterile sources. Using the attA probe, 5 from the 6 investigated CMS genotypes showed identical hybridization patterns to the Petunia CMS line which is used in all commercial sunflower hybrids. Only 1 cytoplasm derived from an open pollination of Helianthus annuus ssp. tschonoskei, known as ANT¹, contained a unique mitochondrial DNA fragment, which is distinguishable from the fertile and sterile Petunia CMS genotypes and from all investigated CMS genotypes. Male fertility restoration and male sterility maintenance of the ANT¹ line are different from the Petunia CMS system, which is a confirmation that a novel CMS genotype in sunflower has been identified.

Sunflower: Mitochondrial genome; Cytoplasmic male sterility; RFLP; attA locus

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flying or creating new sources of male sterility and also to investigate the molecular, biochemical and physiological basis of CMS. Little is known about the molecular determination of CMS in sunflower. In the restriction map of the mitochondrial DNA of sunflower an area of 17 kb, including the atpA gene, is different in the Petiolaris CMS line compared to its fertile analogues [19]. In this paper we present our study of 6 new sunflower CMS genotypes. These genotypes were obtained from intra- or interspecific crosses of Helianthus species or genotypes of sunflower cultivars, and have been characterized for further utilization in breeding programmes.

2. MATERIALS AND METHODS

2.1. Plant material
Plants were grown in the field and leaves, used for DNA isolation, were harvested before flowering. The origins of the different cytoplasmic male sterile genotypes are described in Table 1.

2.2. Isolation of mitochondrial DNA
50 g of leaf material was homogenized in 250 ml ice-cold buffer comprising 0.4 M mannitol, 50 mM Tris-HCl, 3 mM Na2EDTA, 0.2% Poly-Lys, 0.1% bovine serum albumin, 10 mM 2-mercapto-ethanol, pH 8.0. The ruptured cells were filtered through cheesecloth and mirucloth. The debris and crude chloroplast fraction were collected at 3,000 x g for 15 min, followed by a centrifugation at 18,000 x g for 20 min to sediment the mitochondria. Mitochondrial pellets were resuspended in 1 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and lysed with 100 µl 10% SDS and 100 µl 10% Sarkosyl during an incubation of 20 min at 65°C. For protein precipitation 100 µl ice-cold 5 M potassium acetate was added, followed by an incubation for 20 min at 4°C. The precipitated protein complexes were removed by centrifugation at 12,000 x g for 10 min and the supernatants were collected by filtration through cheesecloth for further purification by phenol-chloroform extractions and ethanol precipitation. The nucleic acid pellets were dissolved in 0.4 ml sterile water and treated with RNase 10 mg/ml for 30 min at 37°C. After 2 subsequent phenol-chloroform extractions and a final ethanol precipitation the mtDNA was resuspended in 100 µl TE.

2.3. DNA Analysis
About 2 µg mtDNA was digested with 20 U of restriction enzyme (Bethesda Research Laboratories), fractionated by electrophoresis on a 1% agarose gel and blotted onto Hybond N+ by vacuum blotting (LKB). Hybridization with random priming labeled probes was carried out in 10% dextran sulphate, 1 M NaCl, 1% SDS and 200 µg/ml denatured herring sperm DNA at 60°C. After washing down to 0.1 SSC at 60°C, blots were autoradiographed using Kodak X-Omat AR film.

The probes used in this analysis have been provided by Dr. Toru Terachi, Kyoto Sangyo University, Japan (personal communication). The following mitochondrial probes were used: atpA (1.5-kb HindIII–EcoRI fragment, containing the coding region of subunit A of the ATPase gene of Pisum sativum); cupA (1.9-kb EcoRI fragment, containing the coding region of the cytochrome c oxidase subunit II of Pisum sativum); 18 S + 5 S rRNA (a 3.2-kb BamHI–SalI fragment which contains the 5' upstream region of the 18 S and 5 S rRNA genes and also the tRNA(Met) gene from Tritium aestivum [20]); and 26 S rRNA (a 5.2-kb BamHI–SalI fragment from Tritium aestivum which contains part of the 5' upstream region of the 26 S rRNA gene [21]).

3. RESULTS
Southern blot analyses were performed to investigate the mitochondrial genomes of several new sunflower CMS sources. Digested and electrophoretically separated mtDNA was blotted to nylon membranes and hybridized with different clones of mitochondrial genes. The results show that the atpA probe hybridized to DNA fragments of different sizes in fertile- (F) as compared to sterile- (S) and investigated new CMS genotypes, when digested with the restriction endonucleases BstEII and SalI (Fig. 1). The atpA probe hybridized to a 13.9-kb BstEII fragment and a 4.4-kb SalI fragment from the F line and to a 5.8-kb BstEII and 7.0-kb SalI fragment from the S line. Some weak hybridization can also be observed in addition to the main bands both in the F, S and CMS lines. This probably is due to short repeated sequences as has also previously been reported by Köhler et al. [22]. It was demonstrated that the hybridization pattern of five out of six investigated CMS sources is identical to the Petiolaris sterile genotype, despite the fact that they were developed from different crosses between Helianthus species or by mutagenesis of sunflower cultivars and manifest different morphological and physiological characteristics.

One of the investigated lines, CMS3, showed a different restriction fragment length polymorphism (RFLP) distinguishing both F- and S-lines. The atpA probe hybridized to a 9.2-kb BstEII fragment and a 1.5-kb SalI fragment of CMS3 mtDNA indicated as NS (new sterile). Different hybridization patterns were also found when the atpA probe was hybridized to mtDNA from F-, S- and NS lines after digestion with the enzymes HindIII, BglI, PstI, BstEII/BglI, BstEII/SalI and HindIII/SalI. Fig. 2 presents the RFLPs between F-, S- and NS lines obtained after double digestions with BstEII/BglI-BstEII/SalI and using the atpA probe. The results from Southern blot analysis have been used to

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Table 1

<table>
<thead>
<tr>
<th>Code</th>
<th>Origin</th>
<th>Fertility</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>F</td>
<td>H. annuus</td>
<td>Leroy et al. [27]</td>
</tr>
<tr>
<td>CMS3</td>
<td>S</td>
<td>H. petiolaris X H. annuus</td>
<td>Leroy et al. [27]</td>
</tr>
<tr>
<td>CMS</td>
<td>S</td>
<td>H. argophyllus X H. annuus</td>
<td>Christov [28]</td>
</tr>
<tr>
<td>CMS2</td>
<td>S</td>
<td>γ irradiation of cultivar Hemus</td>
<td>(unpublished results)</td>
</tr>
<tr>
<td>CMS</td>
<td>S</td>
<td>H. annuus ssp. texanus, open pollination</td>
<td>Vrancausiu et al. [26]</td>
</tr>
<tr>
<td>CMS1</td>
<td>S</td>
<td>sonication of cultivar Peredovic</td>
<td>Christov (unpublished results)</td>
</tr>
<tr>
<td>CMS</td>
<td>S</td>
<td>H. scaberulus X H. annuus</td>
<td>Bohorova (unpublished results)</td>
</tr>
<tr>
<td>CMS3</td>
<td>S</td>
<td>H. annuus X H. hirsutus</td>
<td>Bohorova et al. [29]</td>
</tr>
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F. fertile; S. sterile

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create restriction maps of the atpA area in mtDNAs from fertile HAs, sterile CMSs and new sterile CMS sunflower lines (Fig. 3). In this figure it is shown that to the left of the SalI site in the atpA gene the mtDNA organization of the F-, S- and NS lines is colinear. Differences in the mtDNA organization between F-, S- and NS lines are observed to the right of the SalI site. These results emphasize the differences in genome organization between F-, S- and NS lines and exclude the probability of a point mutation in the mitochondrial genome of the NS sunflower line.

In order to further analyze the mtDNA organization in F-, S- and the described CMS lines, and to clarify whether rearrangements took place elsewhere in the mitochondrial genomes, we carried out Southern hybridization analyses with cloned mitochondrial genes from P. sativum and T. aestivum as heterologous probes. The genes used were coxII, 26S rRNA, 18S + 5S rRNA. The results, shown in Fig. 4, indicate that coxII hybridized to the mtDNA from F-, S- and (NS) lines double-digested with BstEII/BglII-BstEII/SalI and BstEII/SalI without any differences among the various lines. No differences were detected either in hybridization patterns using other enzymes like HindIII, BglII, PstI and the other mitochondrial probes, including 26S rRNA, 18S+5S rRNA and coxII. It is obvious that these regions have a similar structure in fertile and all sterile sources and probably are not involved in recombination events associated with CMS in sunflower.

4. DISCUSSION

At present all commercial sunflower hybrids contain the Petiolaris CMS which was found by Leclercq [2]. In order to be able to introduce cytoplasmic diversity into sunflower we analyzed in this study the high molecular weight mtDNA of possible new types of sunflower CMS. Studies were conducted on 6 CMS genotypes and a near-isogenic male sterile and male fertile sunflower line in Southern blot analyses using 10 restriction endonucleases and 5 mitochondrial genes. The atpA probe distinguished between the CMS S- and F-type in the hybridization experiments with all tested restriction enzymes. Out of the 6 investigated CMS genotypes 5 showed identical hybridization patterns indistinguishable from the well known Petiolaris CMS. No specific restorer sunflower lines have been found for the different CMS genotypes. However, some Petiolaris CMS maintainers partially restore male fertility of these sunflower CMS genotypes.

During preparation of this manuscript Crouzillat et al. [23] reported the genetic analysis and molecular basis of 15 sunflower CMS sources which have different origins than the genotypes included in our studies. In agreement with our results they found identical hybridization patterns indistinguishable from the well known Petiolaris CMS. No specific restorer sunflower lines have been found for the different CMS genotypes. However, some Petiolaris CMS maintainers partially restore male fertility of these sunflower CMS genotypes.
probe: \textit{atpA}

Fig. 2. Southern blot analysis of mtDNA isolated from a fertile \textit{H. annuus} (F), a sterile CMS\textsubscript{as} (S) and CMS\textsubscript{bs} (NS) line, obtained from an open pollination of \textit{H. annuus} \textit{ssp. tenuissimus}. The mtDNA, double-digested with \textit{Bst} E\textsubscript{II}/\textit{Bgl} I and \textit{Bst} E\textsubscript{II}/\textit{Sal} I was hybridized with a random priming labeled \textit{atpA} probe. The arrows indicate the CMS-specific polymorphisms between F-, S- and NS lines.

\textit{atpA}, these results lead to the suggestion that probably, by being affected in the \textit{atpA} area the different sunflower CMS genotypes are related.

The organization of the mitochondrial genomes of the \textit{Petunia} CMS genotypes and all studied new CMS lines regarding the 4 other genes which have been examined (\textit{coxII}, 26S \textit{rRNA}, 18S + 5S \textit{rRNA}) is identical to the F-type. According to Crouzillat et al. [23] the genes 26S \textit{rRNA}, 18S + 5S \textit{rRNA} show less variability than those coding for ATPases, but they could still find RFLP differences in 3 groups of CMS cytotypes. Using 3 enzymes and 12 probes per genotype they found 20% RFLPs (using the 26S \textit{rRNA} probe) and 33.4% RFLPs (using the 18S + 5S \textit{rRNA} probe) which differ from the F genotype [23]. In our study we tested 40 different enzyme/probe combinations (except \textit{atpA}) for each CMS genotype and could not find any RFLP. No differences, not only for the 26S \textit{rRNA} and 18S + 5S \textit{rRNA} but also for the \textit{coxII} gene, have been found, which is an indication that these loci are not involved

\textbf{probe: \textit{coxII}}

Fig. 4. Hybridization of mitochondrial gene \textit{coxII} to restriction fragments of sunflower mtDNA from fertile (F), sterile (S) and new sterile (NS) lines, generated by double-digestion with \textit{Bst} E\textsubscript{II}/\textit{Bgl} I, \textit{Bst} E\textsubscript{II}/\textit{Sac} I and \textit{Bst} E\textsubscript{II}/\textit{Sal} I. The hybridization pattern shows no differences between F-, S- and NS lines.
in events associated with CMS. This does not exclude the possibilities of other RFLP differences elsewhere in the mitochondrial genomes, which could be hypothesized as additional deficiencies responsible for CMS. However, recent publications show that in the commonly used CMS sunflower genotype there is a correlation between CMS and co-transcription of a new open reading frame with the *atpA* gene [22,24]. Probably the translation product of this open reading frame is a 16-kDa polypeptide which is suggested to play a role in the CMS phenotype [25]. This also points to the area of the *atpA* locus to be involved in CMS in sunflower.

One of the investigated lines, CMS1, obtained from an open pollination of *H. annuus* ssp. *tenuis* and known as ANT [26], showed a different RFLP distinguishing this NS line from both F- and S genotypes and all new investigated sunflower CMS lines. This NS line was characterized by a complete anther and pollen atrophy and has proven to be stable under various environmental conditions. Classical restorers commonly used in the *Petiolaris* CMS system do not restore fertility of this NS line and up until now no sunflower lines have been found that restore the fertility of the NS line ([26], P. Petrov, unpublished results). These results agree with the molecular characterization and suggest the existence of a novel type of CMS in sunflower, originating from rearrangements in the vicinity of the *atpA* gene. The *atpA* probe in the investigations of Crouzillat et al. [23] detected 8 cytotypes among 15 sunflower cytoplasms; apparently the *atpA* gene is involved somehow in many sunflower CMSs. In order to understand the molecular basis of CMS in sunflower future experiments will concentrate on differences in organisation and expression of genes in the area of the *atpA* locus between F-, S- and NS genotypes.

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