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The CMS-associated 16 kDa protein encoded by *orfH522* in the PET1 cytoplasm is also present in other male-sterile cytoplasms of sunflower

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Abstract

In sunflower plants carrying the PET1 cytoplasm male sterility (CMS) is associated with a new open reading frame (*orfH522*) in the 3′-flanking region of the *atpA* gene and an additional 16 kDa protein. Twenty-seven male-sterile cytoplasms of different origin were studied for the expression of the 16 kDa protein. In addition to the PET1 cytoplasm nine other male-sterile cytoplasms express the CMS-associated protein. These CMS sources originate from different interspecific crosses, from spontaneously occurring male-sterile plants in wild sunflower and from induced mutagenesis. Polyclonal antisera were raised against fusion proteins which contain 421 bp of the 3′-coding region of *orfH522* to verify by immunological methods the identity of the protein in the other CMS cytoplasms. The anti-ORFH522 antiserum showed a positive reaction in the immunoblot with all CMS cytoplasms expressing the 16 kDa protein. Investigations of the mitochondrial DNA demonstrated that all ten CMS cytoplasms which express the 16 kDa protein have the same organization at the *atpA* locus. *OrfH522* is located in the 3′-flanking region of the *atpA* gene. Transcript analyses using *atpA* and *orfH522* as probes gave the same transcript pattern for the investigated CMS cytoplasms, just as for PET1. The MAX1 cytoplasm has an *orfH522*-related sequence but does not synthesize the 16 kDa protein. Using the sodium carbonate treatment the 16 kDa protein proved to be membrane-bound. Computer analyses predict that the hydrophobic N-terminal region of ORFH522 may form a transmembrane helix functioning as membrane anchor.

Introduction

Cytoplasmic male sterility is a trait of higher plants in which pollen production is impaired. However, the vegetative growth and the female fertility are usually normally developed. In different genera, such as *Zea* [13], *Petunia* [34], *Nicotiana* [20], *Sorghum* [2], *Brassica* [17], *Phaseolus* [1] or *Allium* [35], mitochondrially encoded proteins have been correlated with cytoplasmic male sterility. Using antisera directed against synthetic oligopeptides it could be demonstrated in maize

(*Zea mays*) and *Petunia* that new mitochondrial open reading frames, *T-urf13* and *pcf*, respectively, encode these proteins [9, 34]. In *Brassica*, antibodies raised against a glutathione *S*-transferase-ORF138 fusion protein allowed to establish that the CMS-associated 19 kDa protein is the product of the open reading frame *orf138* [17]. In *Phaseolus vulgaris*, antibodies were used to demonstrate that the predicted product of *pvs-orf239* is only detectable within anther tissues of CMS-Sprite, suggesting that PVS-ORF239 associated with male sterility is expressed in a tissue-specific manner [1]. In *Sorghum*, a known mitochondrial gene, *coxI*, modified by recombination events leads to a CMS-associated protein [2]. However, very little is known about the mechanisms leading to male sterility so far. In comparison to other plant species sunflower offers an interesting possibility to investigate cytoplasmic male sterility because more than 30 sources of CMS are known by now [32]. These CMS types either occurred spontaneously or were found as a result of interspecific or intraspecific crosses. Cytoplasmic male sterility has also been induced by mutagenesis using X-ray irradiation or sonification (cited in [42]).

Despite the large number of CMS types which are available in sunflower only the PET1 cytoplasm originating from an interspecific cross of *Helianthus petiolaris* Nutt. and *H. annuus* [30] has been used for commercial hybrid seed production so far [14]. Due to its importance for hybrid breeding the PET1 cytoplasm is the most thoroughly investigated CMS type in sunflower. The organization of the mtDNAs of male-sterile and fertile lines differs only in an area of about 17 kb whereas the remaining 94% are collinear [41]. The rearranged region which is framed by the *atpA* gene and the *cob* gene includes an 11 kb inversion and an insertion of about 5 kb [24, 41]. Due to the 5 kb insertion a new open reading frame (*orfH522*) is created in the 3′-flanking region of the *atpA* gene [24, 28]. *OrfH522* consists of 522 bp of which the first 57 bp are homologous to *orfB* [22]. The deduced molecular weight of the polypeptide encoded by *orfH522* is 19.6 kDa or 14.5 kDa, assuming that the single internal translation start codon is used in the latter case. Two other open reading frames, *orfH708* and *orfH873*, are present in the rearranged area in the fertile lines but no transcripts could be detected [24]. In the PET1 cytoplasm the *orfH708* sequence is disrupted by the 5 kb insertion [44].

Northern hybridizations revealed that *orfH522* is co-transcribed with the *atpA* gene to give an additional larger transcript which can be observed in the male-sterile lines [23, 24, 28]. In etiolated seedlings, fertility restoration does not change the transcript pattern in the hybrids which is identical with that of the male-sterile lines.

OrfH522 is edited at two positions which in both cases leads to an amino acid change [33]. The C-to-U conversions result in an exchange of serine to leucine at position 16 and to a change of arginine to cysteine at position 38. The restoration does not seem to have an influence on the RNA editing. Therefore, it can be concluded that differential RNA editing is not involved in CMS in the PET1 cytoplasm of sunflower [33].

Comparing the mitochondrially encoded proteins only one difference could be detected between the PET1 cytoplasm and the cytoplasm of fertile lines [23, 28]. An additional 16 kDa protein is expressed in the male-sterile lines. Restoration seems to have no influence on the expression of the 16 kDa protein in etiolated seedlings. However, the expression of the 16 kDa protein is reduced anther-specifically in the fertility-restored hybrids [33]. In fertile lines of *H. annuus* and *H. petiolaris* the 16 kDa protein is not detectable [23]. These results indicate that the 16 kDa protein may play a role in creating the CMS phenotype of sunflower.

A large variety of different male-sterile cytoplasms was investigated in order to detect whether the 16 kDa protein is unique to the PET1 cytoplasm or whether any of these other male-sterile cytoplasms have the same mechanism. For immunological investigations of the different male-sterile cytoplasms we raised polyclonal antisera against fusion proteins containing major parts of the *orfH522* open reading frame. In the paper presented here, we report on the reaction of these antisera with mitochondrially encoded proteins of

the PET1 cytoplasm as well as with proteins of the other male-sterile cytoplasms in sunflower. Studies of the organization of the mitochondrial DNA and transcript analyses were performed to confirm the presence of *orfH522* and the expression of this open reading frame in male-sterile cytoplasms which show the 16 kDa protein in the *in organello* translation products. In order to obtain more knowledge about the mechanism leading to male sterility the localization of the CMS-associated 16 kDa protein within the mitochondria of the PET1 cytoplasm was investigated.

Materials and methods

Plant material

The different sources of cytoplasmic male sterility were provided by Dr H. Serieys (INRA, Génétiques et Amélioration des Plantes, Montpellier, France). The investigated CMS cytoplasms and their origins are listed in Table 1. The etiolated seedlings for *in organello* translation were grown under sterile conditions on Murashige and Skoog medium as described in Horn *et al.* [23].

Construction of the expression vector

A 618 bp fragment covering the 3′ end of the *orfH522* from base 91 of the coding region to base 183 downstream of the stop codon was amplified from a mtDNA clone [24] by PCR using the primers PEV23 (ATTTTTTTGA TATCAGTAGC CCG) and MPI21 (AAAGACAACT GCAGGACGGG G) (Roth, Karlsruhe, Germany; Fig. 1A). Due to sequence mismatches of these primers, an *Eco*RV and a *Pst*I restriction site were introduced to the sequence allowing digestion with these enzymes and cloning into pUC18 using standard procedures. An *Asp*718/*Pst*I and an *Asp*718/*Hin*dIII fragment, respectively, were cloned into pQE-31 (Qiagen, Hilden, Germany) giving rise to expression plasmids called pQ424AP and pQ424AH, respectively,

Table 1. Origin of the male-sterile cytoplasms of sunflower investigated (cited in [32, 42]).

Name	FAO Code	Origin	Reference
1. Spontaneously occurring CMS			
H. annuus 367	ANN1	*H. annuus*	Serieys, 1984
H. annuus 519	ANN3	*H. annuus*	Serieys, 1984
H. annuus 521	ANN4	*H. annuus*	Serieys, 1984
NS-ANN-81	ANN5	*H. annuus*	Marinkovic, 1986
AN-67	ANN10	*H. annuus*	Christov, 1992
2. Intraspecific crosses			
Kouban	ANL1	*H. annuus* ssp. *lenticularis*	Anashchenko et al., 1974
Indiana 1	ANL2	*H. annuus* ssp. *lenticularis*	Heiser, 1982
Fundulea 1	ANT1	*H. annuus* ssp. *texanus*	Vranceanu, 1986
3. Interspecific crosses			
Anomalus	ANO1	*H. anomalus*	Serieys, 1986
Argophyllus	ARG1	*H. argophyllus*	Christov, 1990
Argophyllus	ARG2	*H. argophyllus*	Christov, 1990
Argophyllus	ARG3	*H. argophyllus*	Christov, 1992
Bolanderi	BOL1	*H. bolanderi*	Serieys, 1984
Exilis	EXI1	*H. exilis*	Serieys, 1982
Exilis	EXI2	*H. exilis*	Serieys, 1982
CMG2	GIG1	*H. giganteus*	Whelan, 1981
CMG3	MAX1	*H. maximiliani*	Whelan, 1981
Neglectus	NEG1	*H. neglectus*	Serieys, 1986
Fallax	PEF1	*H. petiolaris* ssp. *fallax*	Serieys, 1984
PET/PET	PEP1	*H. petiolaris* ssp. *petiolaris*	Serieys, 1987
Petiolaris	PET1	*H. petiolaris*	Leclercq, 1969
CMG1	PET2	*H. petiolaris*	Whelan, 1980
PHIR 27	PRH1	*H. praecox* ssp. *hirtus*	Christov, 1993
PRUN 29	PRR1	*H. praecox* ssp. *runnioni*	Christov, 1993
Vulpe	RIG1	*H. rigidus*	Vulpe, 1972
4. Mutagenesis			
PEREDOVIK	sonification of 'Peredovik'		Christov, 1993
HEMUS	irradiation of 'Hemus'		Christov, 1993

both containing the coding region from Val-36 to Ser-174 of the predicted amino acid sequence encoded by *orfH522* plus a six-His tag at the N-terminus of the fusion proteins, termed Q424AP and Q424AH, respectively. As mtDNA was used as template the predicted amino acid

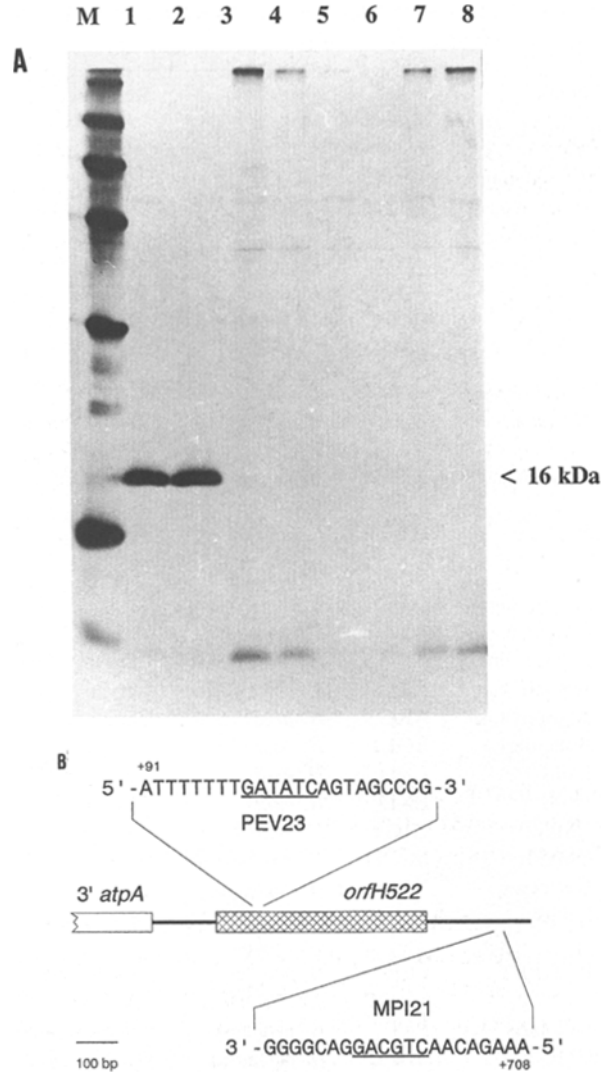

Fig. 1. Characterization of the anti-ORFH522 antiserum raised against the 3'-coding region of *orfH522*. A. Immunoprecipitation of ^{35}S-methionine-labelled mitochondrial protein with anti-ORFH522 antisera (A-Q424AH and A-Q424AP) and pre-immune sera (preA-Q424AH and preA-Q424AP), respectively. The serum samples were diluted 1:10 before use. Antibody-protein complexes precipitated by 'ghosts' of *S. aureus* COWAN A were washed, redissolved, and separated on a 15% SDS-polyacrylamide slab gel. The gel was submitted to fluorography. The arrowhead indicates the position of the mitochondrial 16 kDa protein. Lanes: 1, PET1 with A-Q424AH; 2, PET1 with A-Q424AP; 3, PET1 with preA-Q424AH; 4, PET1 with preA-Q424AP; 5, HA89 with A-Q424AH; 6, HA89 with A-Q424AP; 7, HA89 with preA-Q424AH; 8, HA89 with preA-Q424AP; M, molecular weight marker. B. Construction of the fusion protein used to raise the anti-ORFH522 antiserum. Position of the PCR primer DNA sequences PEV23 (1) and MPI21 (2) relative to *orfH522* in the mtDNA of PET1. The PCR product is a 618 bp fragment covering the complete 3'-coding region of *orfH522* starting with base 91. PEV23 contains an *Eco*RV, MPI21 a *Pst*I restriction site (underlined in both primer sequences) which allowed cloning into the expression plasmid pQE-31 containing a His tag. The fusion protein was overexpressed in *E. coli* and used to raise the anti-ORFH522 antiserum.

change at position 38 (Arg to Cys) due to RNA editing [30] had to be neglected. Amplification and expression of the fusion proteins were obtained in *Escherichia coli* M15. The expression of the proteins was induced by addition of 2 mM IPTG to the culture medium 3 h before harvest. The fusion proteins do not contain the first 57 bp of *orfH522* which show homology to *orfB* so that no cross reaction with a protein encoded by *orfB* or other *orfs*, such as *orfH873* and *orfH708*, which are present in the rearranged area and show a different degree of homology to *orfB* [24], could occur.

Isolation of the fusion proteins

The isolation of the fusion proteins was performed according to the manufacturer's instructions using Ni^{2+}-NTA agarose affinity chromatography (Fig. 1B). The purified proteins were dialysed against phosphate-buffered saline, pH 7.2, and used for the production of antisera in rabbits.

In organello *translation*

Mitochondria of the male-sterile and fertile lines were isolated by differential centrifugation and Percoll density gradient centrifugation. The mitochondrially encoded proteins were labelled by *in organello* translation with ^{35}S-methionine as described by Horn *et al.* [23]. After 90 min protein synthesis was stopped and aliquots were taken to estimate incorporation of ^{35}S-methionine into the proteins. The mitochondria were washed and sedimented. For SDS-polyacrylamide gel electrophoresis (SDS-PAGE) the mitochondria were solubilized in Laemmli sample buffer. Equal

amounts of TCA-precipitable radioactivity were loaded into each lane. The gels were submitted to fluorography as described by Bonner and Laskey [3].

Immunoprecipitation

Pre-immune sera (preA-Q424AP and preA-Q424AH) and anti-ORFH522 antisera (A-Q424AP and A-Q424AH) were diluted 1:10 in phosphate-buffered saline, pH 7.2, and incubated for 60 min with ^{35}S-labelled, mitochondrial *in organello* translation products according to Firestone and Winguth [12]. The resulting protein-antibody complexes were precipitated with 'ghosts' of *Staphylococcus aureus* strain COWAN A provided by W. Schaeg, Institut für Bakteriologie und Immunologie, University of Giessen. The *S. aureus*-bound proteins were washed with TETN250 (25 mM Tris-HCl pH 7.5, 10 mM EDTA, 1% v/v Triton X-100, 250 mM NaCl) twice and once with TE (10 mM Tris-HCl pH 7.5, 5 mM EDTA, 1 mM Pefabloc SC). The samples were immediately solubilized in Laemmli sample buffer and separated on 15% SDS-polyacrylamide slab gels according to Laemmli [27]. The gels were submitted to fluorography as described by Bonner and Laskey [3].

Immunological detection of the proteins on blots

After SDS-PAGE the gels were washed 30 min in transfer buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 5 mM EDTA). The proteins were transferred to Immobilon-P membranes by capillary blotting. Before incubation with antiserum (diluted 1:150 in phosphate-buffered saline/0.05% Tween 20) for 1 h the membranes were saturated with blocking reagents for 1 h. Anti-rabbit antibodies conjugated with alkaline phosphatase (diluted 1:20 000) were used for the colour reaction with nitroblue tetrazoliumchloride and 5-bromo-4-chloro-3-indolyl phosphate. Molecular weights of the detected proteins were estimated by running prestained high molecular weight markers (Gibco BRL).

Analysis of the mitochondrial DNA

Mitochondria were isolated from etiolated seedlings as described by Horn *et al.* [20] but the purification of the mitochondrial fraction by Percoll density gradient centrifugation was omitted. After DNase treatment the mitochondria were centrifuged again at 12 000 rpm for 10 min at 8 °C. The sediments were dissolved in 1 ml lysis buffer (50 mM Tris-HCl pH 7.2, 5 mM EDTA) and immediately frozen at −80 °C. Mitochondrial DNA was isolated by the CTAB procedure as described by Rogers and Bendich [38]. The mitochondrial DNA was digested with *Bgl*II and separated by agarose gel electrophoresis. For Southern hybridization according to Sambrook *et al.* [40] the clone HFE1 carrying the *atpA* gene was cut by *Eco*RI and *Sac*I giving a fragment specific for the 5′-coding region of *atpA*. The *orfH522* specific probe was obtained by cutting the clone HSE1/19 with *Taq*I. Labelling of the probes and ECL detection was performed according to the manufacturer's description (Amersham, Germany).

Northern analysis

Total RNA was extracted using RNA Clean (AGS, Germany) according to the manufacturer's instructions. Two grams of cotyledons from 7-day-old etiolated seedlings were grounded in liquid nitrogen and transferred to centrifuge tubes. Twelve ml RNA Clean (AGS, Germany) and 1.2 ml chloroform were added, mixed and allowed to separate for 30 min at 8 °C. After centrifugation for 15 min at 10 000 rpm at 4 °C the upper phase was transferred into new centrifugation tubes and the RNA precipitated with 6 ml 2-propanol for 30 min at 8 °C.

Samples were centrifuged as before and the supernatants were discarded. The sediments were washed in 2 ml 70% ethanol/DEPC. After centrifugation for 10 min at 10 000 rpm at 4 °C the sediments were dissolved in 200 µl TE/DEPC, transferred into new tubes and precipitated again by adding 500 µl ethanol. After 10 min on ice the

samples were centrifuged at 13 000 rpm for 10 min at room temperature. The sediments were washed with 70% ethanol/DEPC. Finally, the sediments were dissolved in 150 μl TE/DEPC and stored at −80 °C.

For further purification the RNA was treated with phenol:chloroform to eliminate proteins attached to the RNA [40]. After adding an equal volume of phenol/chloroform to the RNA the samples were centrifuged at 13 000 rpm for 15 min at room temperature. The upper phase was transferred to new tubes and precipitated with a double volume of ethanol. After 1 h the samples were centrifuged at 13 000 rpm for 30 min at room temperature. The sediments were washed with 70% ethanol/DEPC and finally the sediments were dissolved in TE/DEPC and frozen at −80 °C.

Formaldehyde gel electrophoresis was done according to a modified method of Sambrook *et al.* [40]. Heat-denatured RNA was fractionated in 1.5% agarose gels containing 0.7 M formaldehyde. The RNA was transferred to Hybond-N + by capillary blot with 20× SSC (3 M sodium chloride, 0.3 M sodium citrate pH 7) overnight and the Hybond-N + was baked at 80 °C for 2 h. The gene probes were labelled by random priming using α^{-35}S- or α^{-32}P-dATP according to the manufacturer's description (Amersham, Germany) and hybridized to blots as described by Sambrook *et al.* [40]. Transcript lengths were estimated using the RNA ladder (Gibco BRL) as marker.

Separation of the mitochondrial proteins into a membrane-bound and a soluble fraction

Mitochondrially encoded proteins labelled with ^{35}S-methionine by *in organello* translation were fractionated into a membrane-bound and a soluble fraction using sodium carbonate [15]. Mitochondria suspended in 50 μl resuspension buffer (10 mM Tricine/KOH pH 7.2, 0.3 M mannitol, 10 mM EDTA) were incubated in 8 ml 100 mM Na_2CO_3, pH 11.5, for 30 min on ice. After ultracentrifugation for 1 h (230 000 × *g*) the proteins in the supernatant (soluble fraction) were precipitated with ice-cold 10% trichloracetic acid for 30 min on ice and centrifuged for 10 min (20 000 × *g*) at 4 °C. After washing with diethylether this sediment as well as the sediment from the ultracentrifugation step were solubilized in Laemmli sample buffer. The samples were heated for 2 min at 80 °C, separated on a 16% SDS-polyacrylamide gel and submitted to fluorography.

Solubilization of the 16 kDa protein from the membranes

After *in organello* translation the washed mitochondrial sediments were incubated with 0.5% (w/v) sodium desoxycholate in a 10 mM Tris-HCl pH 7.5, for 30 min on ice. After ultracentrifugation (230 000 × *g*) for 1 h the proteins of the supernatant containing all solubilized proteins were precipitated using acetone at −20 °C. The sediments obtained by centrifugation (13 000 × *g*) for 10 min were solubilized in Laemmli sample buffer and separated on a 16% SDS-polyacrylamide gel after heating the samples for 2 min at 80 °C. The proteins were visualized by fluorography.

Computational procedures

The hydropathy profile of the amino-acid sequence of ORFH522 derived from the edited *orfH522* sequence was calculated according to the parameter set of Kyte and Doolittle [26] using a window size of 12 residues.

Based on the derived primary structure of ORFH522, secondary structure predictions were carried out using the methods of Chou and Fasman [4], Garnier *et al.* [16], Levin and Garnier [31], Ptitsyn and Finkelstein [36], Rost and Sanders [39] provided by the GENIUS net service of the DKFZ (Deutsches Krebsforschungszentrum, Heidelberg, Germany) and the PredictProtein service of the EMBL (European Molecular Biology Laboratory, Heidelberg, Germany).

Segments which probably have a helical character were further investigated by the hydrophobic moment analysis of Eisenberg *et al.* [10]. The parameters for the hydrophobic moment calculation were taken from Eisenberg's normalized parameter set [11]. The hydrophobic moment was calculated for segments with a size of 18 residues as this represents the lower limit for a membrane-spanning helical element.

Results

Expression of the 16 kDa protein in different allo- and autoplasmic male sterility sources in sunflower

Twenty-seven of the 33 CMS-inducing cytoplasms described to date in the genus *Helianthus* (Table 1), 27 were investigated on protein level. The mitochondrially encoded proteins labelled with ^{35}S-methionine were investigated with regard to the expression of the 16 kDa protein which is correlated with CMS in the PET1 cytoplasm (Fig. 2 and Fig. 4A). Ten of the CMS cytoplasms, including PET1, express a protein of approximately 16 kDa (Table 2). These cytoplasmic male-sterile germplasms have been produced by either different interspecific crosses involving *H. argophyllus*, *H. neglectus*, *H. exilis*, *H. petiolaris*, *H. anomalus*, and two subspecies of *H. praecox*, or by mutagenesis of two maintainer lines for the PET1 cytoplasm ('Hemus' and 'Peredovik'). In addition, one of the CMS types that arose spontaneously (ANN10) expresses the 16 kDa protein.

Identification of the CMS-associated protein using the anti-ORFH522 antiserum in different CMS cytoplasms

In immunoprecipitation experiments the anti-ORFH522 antiserum specifically precipitated one protein of ca. 16 kDa from the mitochondrially encoded proteins of the PET1 cytoplasm which had been radioactively labelled by *in organello* translation (Fig. 1A). According to its molecular weight this protein corresponds to the protein predicted by the sequence of *orfH522* with 19.6 kDa or 14.5 kDa (if the internal start codon is used), respectively. In the mitochondria of the fertile lines no cross-reaction with any mitochon-

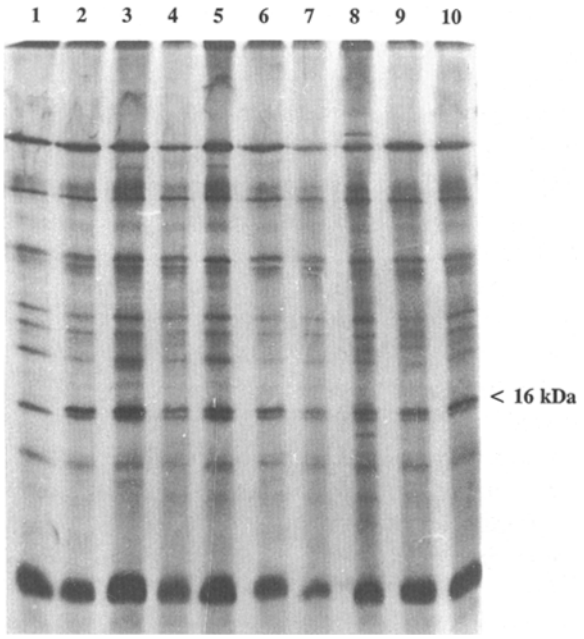

Fig. 2. Expression of the CMS-associated 16 kDa protein in male-sterile cytoplasms of different origins. Mitochondrial proteins from etiolated seedlings were labelled with ^{35}S-methionine by *in organello* translation and separated on a 16 % SDS-polyacrylamide gel. The 16 kDa protein which is associated with CMS in the PET1 cytoplasm is marked by an arrowhead. Lanes: 1, HA89; 2, PET1; 3, EXI2; 4, NEG1; 5, ANN10; 6, PRR1; 7, ARG1; 8, PEREDOVIK; 9, PRH1; 10, HEMUS; M, molecular weight marker.

Table 2. CMS cytoplasms in sunflower expressing the 16 kDa protein.

CMS cytoplasm	Origin
ANN10	*H. annuus*-E-067
ANO1	*H. anomalus*
ARG1	*H. argophyllus*-E-006
EX12	*H. exilis*
HEMUS	*H. annuus*, irradiation
NEG1	*H. neglectus*
PEREDOVIK	*H. annuus*, sonification
PET1	*H. petiolaris*
PRH1	*H. praecox hirtus*
PRR1	*H. praecox reunioni*

drially encoded protein was observed. The immunoprecipitated 16 kDa protein is identical with respect to its molecular weight to the protein that has been associated with CMS in the PET1 cytoplasm by comparing the *in organello* translation products [23, 28]. Since this protein represents the only detectable difference between male-sterile and fertile lines in the expression of the mitochondrially encoded proteins, the result indicates that the *orfH522*-encoded protein and the unique mitochondrial protein are the same.

To determine whether the 16 kDa protein observed in the other CMS cytoplasms is identical to the CMS-associated protein of the PET1 cytoplasm immunological studies were conducted. In immunoblots using the anti-ORFH522 antibodies all of these CMS-inducing cytoplasms showed a positive reaction (Figs. 3 and 4B). However, these reactions were remarkably weaker in intensity than those with the 16 kDa protein of the PET1 cytoplasm. This might indicate that these proteins are slightly different in amino acid composition or are expressed in a reduced amount. Nevertheless, there is a clear correlation between cytoplasmic male sterility and the presence of this protein. In some immunoblots, a second band much weaker in intensity and with a lower molecular weight than the main signal can be observed. This product might be due to processing or degradation. A protein corresponding to this signal is not detectable in the *in organello* translation products. The reason for this might be either that the protein is not labelled, since it does not contain a methionine, or that it is labelled too weakly so that it cannot be detected or it is a nuclear encoded protein. However, it cannot be excluded that these two proteins represent the 19.6 kDa and the 14.5 kDa proteins, respectively.

It is also remarkable that interspecific crosses involving, for example, *H. exilis* or *H. argophyllus* allow the development of different CMS forms as EXI2 expresses the 16 kDa protein whereas EXI1

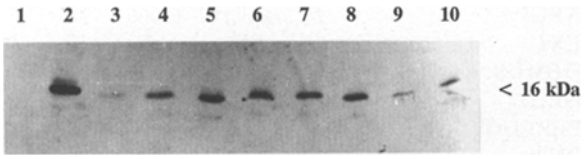

Fig. 3. Immunoblot of total mitochondrial proteins of different CMS sources probed with the anti-ORFH522 antiserum. Mitochondrial proteins (40 μg per lane) were separated on a 16% SDS-polyacrylamide gel. The proteins were transferred to Immobilon-P membrane and incubated with the anti-ORFH522 antiserum diluted 1:150. The 16 kDa protein is marked by an arrowhead. Lanes: 1, HA89; 2, PET1; 3, EXI2; 4, NEG1; 5, ANN10; 6, PRR1; 7, ARG1; 8, PEREDOVIK; 9, PRH1; 10, HEMUS.

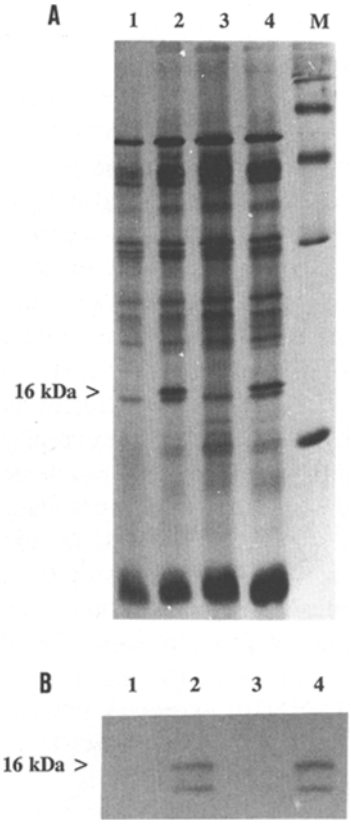

Fig. 4. Analysis of mitochondrial proteins of MAX1 and ANO1 by *in organello* translation and immunoblotting. A. *In organello* translation products obtained by labelling the mitochondrially encoded proteins with ^{35}S-methionine. The proteins were separated on a 16% SDS-polyacrylamide gel and visualized by fluorography. The 16 kDa protein associated with CMS in PET1 is marked by an arrowhead. Lane: 1, HA89; 2, PET1; 3, MAX1; 4, ANO1; M, molecular weight marker. B. Immunological investigation of total mitochondrial proteins by probing with the anti-ORFH522 antiserum. The proteins (40 μg per lane) were separated on a 16 % SDS-polyacrylamide gel. The antiserum was diluted 1:150. Lanes: 1, HA89; 2, PET1; 3, MAX1; 4, ANO1.

does not. On the other hand, in both EXI2 and ARG1, originating from different interspecific crosses, the 16 kDa protein is detectable.

Another CMS source, MAX1 which was obtained by an interspecific cross of *H. maximiliani* and *H. annuus*, has been investigated closely. This MAX1 cytoplasm shows a region with 93% homology to *orfH522* [19]. This *orfH522*-related sequence is missing the first base pair of the start codon. MAX1 does not express the 16 kDa protein (Fig. 4A,B) which is in accordance with the described sequence analysis. The predicted protein of *orfM160*, a new open reading frame starting 48 bp downstream of the original *orfH522* start codon in MAX1 [19], would have a sequence-deduced molecular mass of ca. 17.8 kDa, or either 15.7 or 14.9 kDa if one of the two internal start codons is used. The gene product of *orfM160* would be predicted to have 81.2% homology to the *orfH522*-encoded protein. This means that the polyclonal antibody prepared against the fusion protein containing the last 421 bp of *orfH522* should also recognize the predicted protein of *orfM160*. No protein of the estimated molecular weight could be observed among the *in organello* translation products or could be detected by the anti-ORFH522 antiserum (Fig. 4A,B), respectively. Mitochondria isolated from etiolated seedlings carrying the MAX1 cytoplasm do not express a protein corresponding to this region of the mtDNA.

Organization of the mitochondrial DNA at the atpA *locus*

Southern analyses were performed using *atpA* and *orfH522* as probe against mitochondrial DNA digested with *Bgl*II to investigate the organization of the mitochondrial DNA of the male-sterile cytoplasms that express the 16 kDa protein (Table 2). As *Bgl*II cuts within the *atpA* probe used for hybridization the experiments give separate information about the 5′ region as well as about the 3′-region of the *atpA* gene (Fig. 5). The hybridization signal of 1.2 kb is detectable in all male-sterile cytoplasms expressing the 16 kDa protein and the fertile line HA89, respectively. This signal demonstrates that the organization of the mitochondrial DNA in the 5′ region of the *atpA* gene is identical in the investigated lines. However, for the 3′ region different signals were obtained for the fertile and the male-sterile lines. A *Bgl*II fragment of 7.1 kb is characteristic of the fertile line whereas all male-sterile cytoplasms expressing the 16 kDa protein show the same 4.9 kb fragment. Southern hybridizations using *orfH522* as probe demonstrate that this 4.9 kb fragment also contains the *orfH522* (Fig. 5). All cytoplasms that express the 16 kDa protein have the same organization at the *atpA* locus as the PET1 cytoplasm. The *orfH522* is located in the 3′-flanking region of the *atpA* gene.

Transcript analyses of the atpA *gene and* orfH522

Northern hybridizations were performed to investigate the expression of *atpA* and *orfH522* in CMS cytoplasms which show the 16 kDa protein in the *in organello* translation pattern. Transcript analyses using the *atpA* gene as probe (Fig. 6A) revealed that the male-sterile cytoplasms, PET1, ANN10, ANO1, EXI2 and ARG1, expressing the 16 kDa protein show an additional larger transcript of 2900 nt besides the main transcript of 2100 nt present in the fertile line HA89. The larger transcript has been demonstrated to represent a cotranscript of the *atpA* gene and *orfH522* in PET1 [24]. The transcript pattern of the *atpA* gene is identical in the investigated CMS sources.

Using *orfH522* as probe (Fig.6B) no transcripts were detected in the fertile line HA89 but all male-sterile cytoplasms investigated showed the same transcript pattern as the PET1 cytoplasm. These studies included CMS sources originating from interspecific crosses and the spontaneously occurring CMS source. The two CMS cytoplasms due to mutagenesis were not investigated. The larger transcript of 2900 nt detected with *atpA* can also be observed with *orfH522* as probe. The northern analyses demonstrate that in these male-sterile lines *orfH522* is cotranscribed with the *atpA* gene, as in PET1.

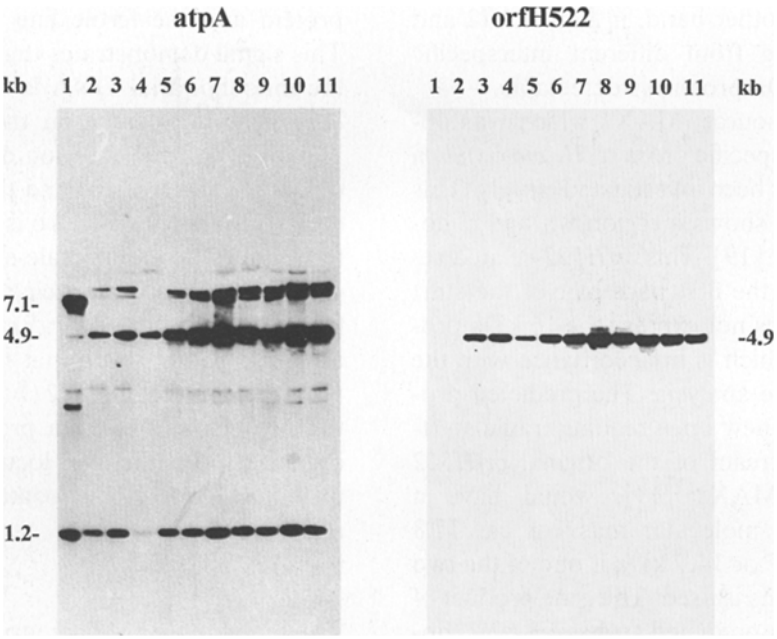

Fig. 5. Organization of the mitochondrial DNA at the *atpA* locus in the fertile line HA89, the male-sterile line PET1 and nine other male-sterile lines expressing the 16 kDa protein. Southern hybridizations were performed using *atpA* (digested with *EcoR*I/*Sac*I) and *orfH522* (digested with *Taq* I) as probes against mitochondrial DNA digested with *Bgl* II. Lanes: 1, HA89; 2, PET1; 3, EXI2; 4, NEG1; 5, ANN10; 6, PRR1; 7, ARG1; 8, PEREDOVIK; 9, PRH1; 10, HEMUS; 11, ANO1.

Localization of the 16 kDa protein in the mitochondria

The intramitochondrial localization of the CMS-associated 16 kDa protein expressed in male-sterile lines carrying the PET1 cytoplasm is important to understand the mechanism being responsible for CMS in sunflower. Using the sodium carbonate treatment mitochondrial proteins can be separated into a soluble and a membrane-bound fraction as membranes and membrane-associated proteins are insoluble in sodium carbonate solution (pH 11.5). Most of the *in organello* translation proteins are membrane-associated (Fig. 7). The 16 kDa protein correlated with CMS was only present in the membrane fraction. Even after prolonged exposition no traces of the 16 kDa protein could be detected in the soluble fraction (data not shown). Among the proteins of the soluble fraction, the α subunit of the F_1-ATPase and a 40 kDa protein, which could be a protein corresponding to the ribosomal protein var-1 of yeast [6], are present in significant amounts. Both proteins have been described as soluble mitochondrial components [29]. The association of the F_1 complex and the integral membrane F_0 complex of the F_1-ATPase is probably partially weakened due to the high concentration of EDTA in the resuspension buffer. However, it is not possible to localize the 16 kDa protein in the inner or the outer mitochondrial membrane by this method.

In order to purify the 16 kDa protein it has to be solubilized from the membrane. As detergents like SDS are difficult to handle in an isolation procedure using, for example, gel filtration, sodium desoxycholate was tested as detergent. At a concentration of 0.5% (w/v) sodium desoxycholate was able to solubilize the 16 kDa protein in a nearly quantitative manner (Fig. 7).

Secondary structure analysis predicts a membrane anchor in the N-terminus of ORFH522

Computer analyses based on the primary structure of ORFH522 were performed to find out

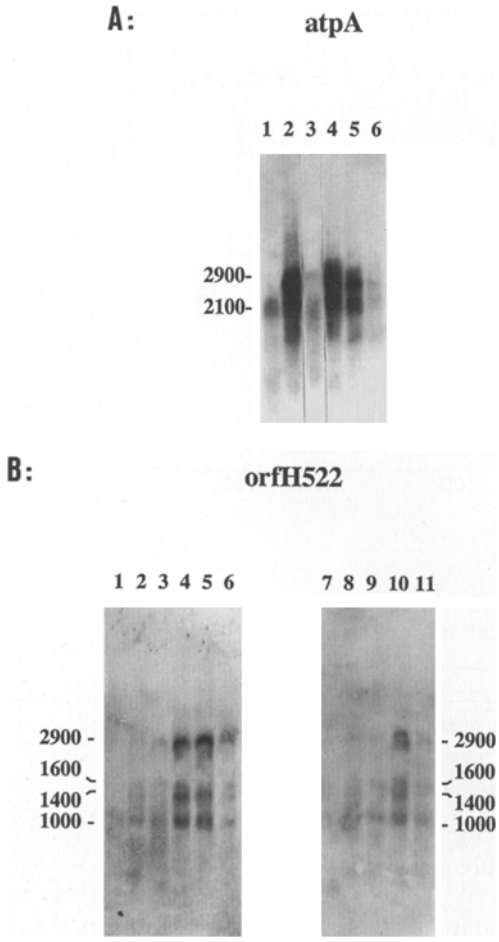

Fig. 6. Transcript analysis of the *atpA* gene and *orfH522* in male-sterile cytoplasms expressing the 16 kDa protein and the fertile line HA89. Total RNA (10 μg per lane) from etiolated seedlings of the fertile line HA89 and male-sterile lines carrying CMS cytoplasms of different origin were hybridized to *atpA* and *orfH522* as probes. Transcript lengths are designated in nucleotides. A. Transcript pattern obtained by hybridization using the *atpA* gene as probe. Lanes: 1, HA89; 2, PET1; 3, ANN10; 4, ANO1; 5, EXI2; 6, ARG1. B. Northern hybridizations using *orfH522* as probe. Lanes: 1, HA89; 2, PET1; 3, ANN10; 4, ANO1; 5, EXI2; 6, ARG1; 7, HA89; 8, PET1; 9, NEG1; 10, PRH1; 11, PRR1.

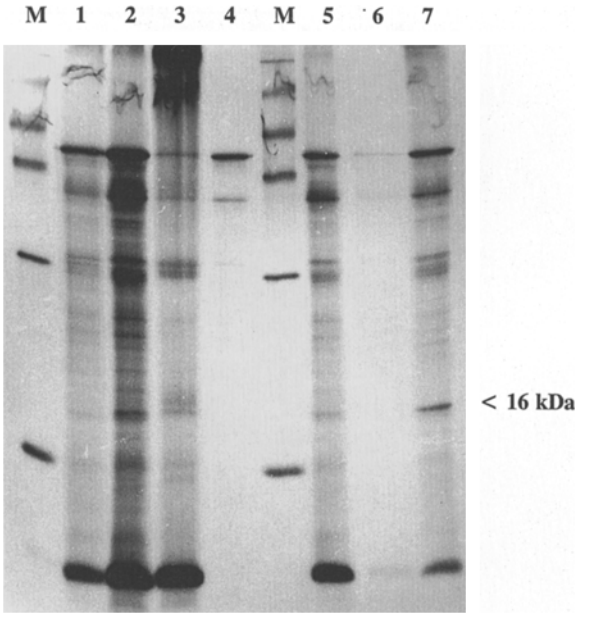

Fig. 7. Localization of the 16 kDa polypeptide within the mitochondria. Mitochondrial proteins labelled with ^{35}S-methionine of the male-sterile cytoplasm PET1 were either treated with 10 mM sodium carbonate (pH 11.5) and separated by ultracentrifugation into a membrane-bound and a soluble fraction or extracted with 0.5% (w/v) sodium desoxycholate in order to solubilize the membrane proteins from the inner and outer membrane. Insoluble fragments were sedimented by ultracentrifugation. The proteins were separated on a 16% SDS-polyacrylamide gel. In the fluorography the 16 kDa protein is marked by an arrowhead. Lanes: 1, untreated mitochondria of the fertile line HA89; 2, untreated mitochondria of PET1; 3, membrane-bound fraction of PET1 after incubation with sodium carbonate; 4, soluble fraction of PET1 after sodium carbonate treatment, precipitated by acetone; 5, untreated labelled mitochondrial proteins of PET1; 6, sediment after the treatment of mitochondria with 0.5% sodium desoxycholate; 7, solubilized proteins in the supernatant precipitated with acetone; M, molecular weight marker.

which part of ORFH522 might be membrane-associated. The hydropathy profile of ORFH522 (Fig. 8A) reveals a hydrophobic region in the N-terminus (residues 23–45). Secondary structure predictions localized segments with a high probability for helical organization (Fig. 8B). Predicted helices with a length of more than 18 residues were used to construct a consensus prediction of all applied methods resulting in three helical regions. Within these predicted helical regions segments with a size of 18 residues were analysed according to the hydrophobic moment plot of Eisenberg *et al.* [10]. The values of the hydrophobic moment of each 18-residue segment were plotted as a function of the mean hydrophobicity of the segments (Fig. 8C). Helices with high hydropathy accompanied by low amphiphilic character appear in the transmembrane region of

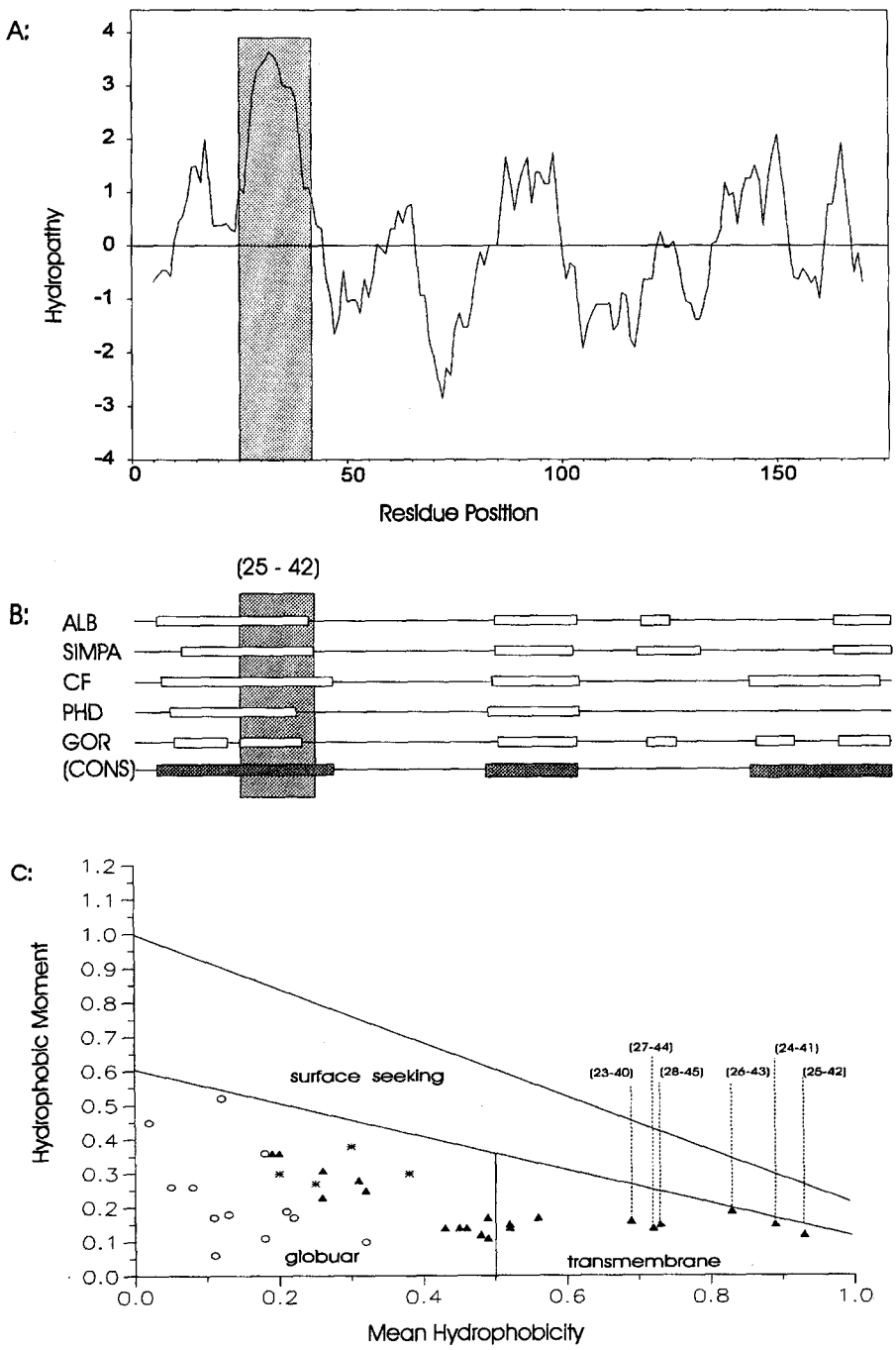

Fig. 8. Computer analysis of the amino acid sequence of ORFH522 derived from the edited *orfH522* sequence. A. Hydropathy profile of the derived amino acid sequence of ORFH522 according to the values of Kyte and Doolittle [26]. The shaded bar marks the proposed membrane anchor region. B. Secondary structure predictions following the methods of Ptitsyn and Finkelstein (ALB) [36], Levin and Garnier (SIMPA) [31], Chou and Fasman (CF) [4], Garnier *et al.* (GOR) [16], Rost and Sander (PHD) [39]. The consensus of the predicted helices is shown in shaded bars (CONS). C. Hydrophobic moment plot for the 18-residue segments of the consensus of the predicted helices according to Eisenberg *et al.* [10]. Helix 1 = ▲, Helix 2 = *, Helix 3 = ○.

the plot which indicates a high affinity for the membrane interior. All calculated segments which can be found in the transmembrane part belong to the sequence between the residues 23 to 45 of ORFH522. The segment (residue 25 to 42) with a mean hydrophobicity of 0.93 and a hydrophobic moment of 0.12 represents the best candidate for a transmembrane helix according to the hydrophobic moment analysis. It exactly matches the required length of 18 residues which is necessary for a membrane-spanning element corresponding to 5 helix turns. Based on these results we suggest that this region (residue 25 to 42) represents a putative membrane anchor of ORFH522.

Discussion

In sunflower plants carrying the PET1 cytoplasm the CMS phenotype correlates with the presence of a new open reading frame (*orfH522*) in the mitochondrial DNA [24, 28] and an additional 16 kDa protein in the mitochondrial *in organello* translation products [23, 28]. Screening 27 auto- and alloplasmic male sterility sources in sunflower, which are different by their origin, the 16 kDa protein associated with CMS in the PET1 cytoplasm was detected in the *in organello* translation products of nine other CMS sources in addition to the PET1 cytoplasm. To verify that the 16 kDa protein present in these CMS cytoplasm is the CMS-associated protein of PET1 a polyclonal antiserum was raised against a fusion protein containing the last 421 bp of *orfH522*. In PET1, this anti-ORFH522 antiserum specifically immunoprecipitated one protein from the *in organello* translation products corresponding to the CMS-associated 16 kDa protein. This confirmed that *orfH522* encodes for the 16 kDa protein which is in accordance to Monéger *et al.* [33] who used an antiserum against a synthetic oligopeptide to show this.

Investigating the other CMS cytoplasms that express a 16 kDa protein the anti-ORFH522 antiserum proved that the corresponding proteins in these CMS cytoplasms were indeed very similiar to the 16 kDa protein of PET1 as the anti-ORFH522 antiserum showed a clear reaction with the 16 kDa proteins in these other CMS sources although the reaction was slightly weaker.

As the 16 kDa protein represents the translation product of *orfH522* it can be expected that *orfH522* or very closely related *orfs* will be present in the mitochondrial DNA of the other male-sterile cytoplasms expressing the 16 kDa protein. For four of the CMS cytoplasms, ARG1, PEREDOVIK, HEMUS [42] and ANO1 [7, 8], indirect evidence exists that *orfH522* might be located on the same site as in the PET1 cytoplasm, i.e. next to the *atpA* gene. In Southern hybridizations using the *atpA* gene as probe these four CMS cytoplasms show the pattern that is typical of the PET1 cytoplasm, where *orfH522* is located in the 3′-flanking region of the *atpA* gene. In fertile *H. annuus* this pattern is different and no homology to *orfH522* can be observed [24].

Southern hybridizations using the *atpA* gene and *orfH522* as probes presented in this paper here give a clear picture about the organization of the *atpA* locus. These investigations on mtDNA level demonstrate that in all male-sterile cytoplasms expressing the 16 kDa protein *orfH522* is located in the 3′-flanking region of the *atpA* gene, just as in PET1. The organization of the 5′-flanking region of the *atpA* gene seems to be identical in the fertile line HA89 and the 10 investigated male-sterile cytoplasms.

Transcript analyses using *atpA* and *orfH522* as probes confirmed the expression of this region. Transcript patterns observed were the same for all investigated CMS sources expressing the 16 kDa protein as for PET1. The larger transcript of 2900 nt representing the cotranscript of the *atpA* gene and *orfH522* is detectable in these male-sterile lines but not in the fertile line HA89. However, it remains difficult to explain how different interspecific crosses, mutagenesis and spontaneously occurring CMS, respectively, seem to lead to the same CMS-associated region. The organization of the mtDNA observed in PET1 may represent an ancient configuration or independent events may have led to the creation of *orfH522* or related *orfs*, respectively. According to

Monéger *et al.* [33] the rearranged organization of the *atpA* locus observed in the PET1 cytoplasm is present at a low level in the mtDNA of the fertile line, as a sublimon. This sublimon could have been amplified due to the interspecific cross rather than being the result of a novel recombination event induced by nucleo-cytoplasmic incompatibility. This is, however, in contrast to results obtained by Rieseberg *et al.* [37]. Screening 1200 plants representing 55 accessions of *H. annuus* and 26 accessions of *H. petiolaris* with specific PCR primers no PET1 cytotype was observed in natural populations of either *H. annuus* or *H. petiolaris*.

It is especially interesting to note that interspecific crosses, including *H. exilis* or *H. argophyllus*, resulted in different CMS types, one expressing the CMS-associated 16 kDa protein, the other not. All the CMS sources expressing the 16 kDa protein belong to species of the section Annui within the genus *Helianthus* whereas MAX1, which contains an *orfH522*-related sequence that is not expressed, belongs to the section Divaricati.

If all of the CMS cytoplasms expressing the 16 kDa protein had the same CMS mechanism their maintainer and restorer lines should be the same as for the PET1 cytoplasm. Few results concerning the characterization of the new CMS cytoplasms by different restorer and maintainer lines have been published [5, 21]. ARG1 as well as ANN10 are fully restored by lines known as restorers of the PET1 cytoplasm. A genetic differentiation between PET1 and ANO1 seems to be possible by the male fertility restoration/male sterility maintenance pattern using 16 lines [7, 8]. Fifteen lines showed an identical behaviour whereas the line PR4 proved to be a restorer of PET1 but a maintainer of ANO1. The authors assumed that a supplementary deficiency responsible for CMS could exist in ANO1 as this cytoplasm shows RFLPs not only with the *atpA* gene but also with *coxIII*, *cob* and *atp9* genes as probes [7, 8]. The principle component analysis of the RFLP study investigating 15 CMS sources allowed to distinguish 13 groups based on differences in the mtDNA organization [8]. DNA molecular diversity occurs both within and between the *Helianthus* species from which the male-steriles originated.

Another CMS line, ANT1 named CMS3 by Spassova *et al.* [43], does not express the 16 kDa protein. However, the *atpA* region is rearranged. In ANT1 the *orfb-coxIII* locus is located immediately 3′ to the *atpA* gene whereas in the fertile cytoplasm these loci are about 60 kb apart. This DNA rearrangement probably involves the 261 bp repeat, as in PET1.

With regard to the mechanism leading to CMS in the PET1 cytoplasm the intramitochondrial localization of the CMS-associated 16 kDa protein may give a first hint for its function. According to the results obtained by the sodium carbonate treatment the 16 kDa protein represents a membrane-associated protein. Computer analyses support this result. The hydrophobic N-terminal region is predicted to form a transmembrane helix as it has the required length of 18 residues and appears in the transmembrane part of the hydrophobic moment plot of Eisenberg *et al.* [10]. In rapeseed cybrids with 'Ogura' cytoplasm the CMS-associated 19 kDa protein encoded by *orf138* also proved to be membrane-bound [17]. In addition, the hydrophobicity plot also predicts a hydrophobic N-terminus, as in PET1. In *Phaseolus vulgaris*, the product of the CMS-associated *pvs-orf239* contains a hydrophobic N terminus commonly found in membrane-bound proteins, too [1]. Also, in maize cms-T the CMS-associated 13 kDa protein is localized in the membrane fraction being an integral part of the inner mitochondrial membrane [18, 25]. In the presence of the T-toxin the 13 kDa protein seems to exist in an oligomeric form of 2–4 subunits in the inner mitochondrial membrane forming a channel which increases the membrane permeability [25]. However, membrane association does not seem to be a general feature of CMS as the 25 kDa protein in *Petunia* is found in the membrane fraction as well as in the soluble fraction [34].

The association of the 16 kDa protein with the membrane fraction is a first hint on the way to understand how the *orfH522* translation product

may affect mitochondrial function during microsporogenesis leading to pollen abortion in sunflower with the PET1-type mitochondria. A comparison of the different CMS sources expressing the 16 kDa protein will give a closer insight into the molecular mechanism leading to CMS.

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