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ORIGINAL PAPER

Mary M. Robison · David J. Wolyn

Petaloid-type cms in carrot is not associated with expression of *atp8* (*orfB*)

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Abstract Two different *atp8* reading frames with divergent C-terminal extensions were identified in a sterility-inducing mitochondrial type (mitotype) of carrot; examination of three fertility-inducing mitotypes revealed *atp8* reading frames that were either terminated at a position conserved among other plant *atp8* genes or continued with unique C-terminal extensions. Similarities between the C-terminal extensions suggest ancient common ancestry, but the level of sequence divergence implies lack of functional conservation. Northern analysis indicated that the C-terminal extensions are transcribed and are present within the primary *atp8* transcripts. Western analysis revealed that the products of the two extended *atp8* reading frames encoded by the sterility-inducing mitotype are significantly smaller than those predicted by complete translation of their C-terminal extensions, but are slightly larger than the products from unextended reading frames. No alterations were observed in *atp8* transcripts or proteins isolated from flowers of the sterility-inducing mitotype that were phenotypically fertile as a result of segregation for nuclear fertility-restoring Ms or Rf alleles, and thus, there is no evidence that expression of *atp8* is directly involved in the petaloid cms phenotype in carrot.

Abbreviations cms: cytoplasmic male sterility · mt: mitochondrial · mtDNA: mitochondrial DNA · mitotype: mitochondrial type

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M. M. Robison (✉) · D. J. Wolyn
Department of Plant Agriculture, Bovey Bldg.,
University of Guelph, Guelph, ON N1G 2W1 Canada
E-mail: mrobison@uoguelph.ca
E-mail: dwolyn@uoguelph.ca
Fax: +519-7670755

Introduction

Cytoplasmic male sterility (cms) is the maternally-inherited inability of a plant to produce functional pollen. Cms in carrot (*Daucus carota*) may manifest as two very different phenotypes, brown anther or homeotic-like. Brown anther sterility is characterized by developmental arrest of stamens, while homeotic-like describes the replacement of stamens with structures resembling other floral organs, such as sepal or petal-like structures ('petaloid') or carpel-like structures ('carpeloid') (Nothnagel et al. 2000; Linke et al. 2003). The expression of petaloidy by sterility-inducing cytoplasms in carrot is mediated by nuclear loci, including (1) Maintainer-of-sterility (Ms) wherein dominant alleles promote expression of the petaloid phenotype (Morelock 1974), and (2) Restorer-of-fertility (Rf) wherein dominant alleles promote conversion of petaloid organs to fertile stamens (Wolyn and Chahal 1998).

In many plant species, the molecular determinants of the cms phenotype are chimeric mitochondrial (mt) open reading frames, of which the transcripts or translation products are suppressed in the presence of nuclear restorer alleles (Schnable and Wise 1998). Petaloid-type cms in carrot was recently proposed by Nakajima et al. (2001) to be closely associated with an *atp8* (*orfB*) reading frame that possesses a particular, long C-terminal extension (*orfB*-CMS), but they derived their conclusions from a PCR-based survey of *atp8* alleles in which not all products were sequenced and, as a result, may have been incorrectly identified. Concurrently, Bach et al. (2002) and our group independently determined the genomic sequences of *atp8* from a sterility-inducing mitochondrial type (mitotype) [*atp8*-Sp1 (AY007819) and *atp8*-Sp2 (AY007821), (Bach et al. 2002); *atp8*-1 (AY061992 and AY061993) and *atp8*-2 (AY061991), (Robison and Wolyn 2002)]; both sets of data indicated that another allele of *atp8* (*atp8*-Sp2/*atp8*-2) is present in sterility-inducing mitotypes, in addition to *orfB*-CMS (= *atp8*-Sp1/*atp8*-1), and that it also has a

unique C-terminal extension. Nakajima et al. (2001) had amplified this second allele from sterility-inducing mitotypes but most likely misidentified it as *orfB*-F2, an allele with its own unique C-terminal extension found in certain fertility-inducing mitotypes [= *atp8*-N (Bach et al. 2002)], because the primers used in amplification did not differentiate between *atp8*-2 (*atp8*-Sp2) and *orfB*-F2 (*atp8*-N). Thus, alleles of *atp8* with unique C-terminal extensions are found in both sterility-inducing and fertility-inducing mitotypes, and two different alleles with unique C-terminal extensions are present in the same sterility-inducing mitotype.

We present here sequences for new extended and unextended *atp8* alleles from two additional fertility-inducing mitotypes and demonstrate that reading frames for *atp8* in carrot encode a wide variety of C-terminal extensions that probably share a common sequence ancestry but have diverged in association with particular mitotypes. We further examine the possibility that the extended versions of *atp8* in the sterility-inducing mitotype might be causally associated with petaloid cms in carrot by determining if transcripts and/or translation products of this gene are suppressed or altered in the presence of nuclear restorer alleles; we show that neither the size nor the quantity of *atp8* transcripts or translation products is altered in flowers that have been restored to fertility as a result of the expression of nuclear fertility-restoring Ms or Rf alleles, and therefore conclude that, despite the assertions of Nakajima et al. (2001), there is presently no evidence that the mitochondrial determinant of petaloid cms in carrot is *atp8*.

Materials and methods

Plant material

Mitotypes SW3, FW2, FW4 and FG17 refer to unique mitochondrial types identified by mtDNA restriction patterns to be associated with carrot lines W259A, W33B, and W259B (obtained from W. Gabelman, University of Wisconsin, Wisconsin, USA), and GBKF (collected from a wild population near Guelph, ON, Canada), respectively; the S or F in each label identifies the mitotype as sterility-inducing or fertility-inducing, respectively. Full-sib families with the SW3 mitotype segregating 1 sterile:1 fertile were developed by (1) crossing Msms (sterile) × msms (fertile) segregants from the 1558S × W93B cross reported by Morelock (1974) (obtained from P. Simon, University of Wisconsin), and (2) crossing rfrf (sterile) × Rfrf (fertile) segregants from crosses between SW3-containing lines and restorer line W266D (obtained from W. Gabelman, University of Wisconsin).

DNA and RNA isolation

Total DNAs were isolated from leaves and immature flowers by a CTAB miniprep protocol (Rogers and Bendich 1994). Total RNAs were isolated from immature flowers with the RNeasy Plant Mini Kit (Qiagen).

Southern and northern hybridization

Restriction enzyme digestion, agarose gel electrophoresis and Southern blotting were according to standard protocols (Sambrook et al. 1989). DNA probes were synthesized by incorporation of DIG-dUTP (Roche) during PCR amplification. Hybridizations, detections and probe removals were performed as recommended for labeling with digoxigenin (Roche).

RNA samples were denatured and electrophoresed in formaldehyde gels which were subsequently blotted in 20×SSC. RNA antisense probes were synthesized by incorporation of DIG-UTP during in vitro transcription from PCR-derived templates (Maxiscript, Ambion) that were created using similar primer pairs as those used in the creation of DNA probes described below, but with the addition of a T7 promoter to the primer at the 3′ end of the sequence. Hybridizations and detections were performed as recommended for labeling with digoxegenin (Roche).

The templates and primer pairs used in the amplification of DNA probes were as follows:

1. *atp8* core, SW3 DNA (coreF - 5′ CACAATTCTTCTGGTCATGCC 3′ and coreR - 5′ TCGACGGCGTTACACCATTGG 3′);
2. *atp8*-1 extension, SW3 DNA (8-1F - 5′ GATTATATGACTCCTTCTTTCAC 3′ and 8-1R - 5′ CGATATAGACTCTATTTTGA 3′);
3. *atp8*-2 extension, SW3 DNA (8-2F - 5′ CTTAGAGACCTCAATCTCTTAC 3′ and 8-2R - 5′ CGATATAGACTCTATTTTTC 3′);
4. FW2 *atp8* extension, FW2 DNA (2extF - 5′ CTTATATACCCCTATGTCC 3′ and 2extR - 5′ CAGAAAGGGGAGGTCTGG 3′);
5. FG17 *atp8* extension, FG17 DNA (17extF - 5′ CGCATATGCCTCTATCTATG 3′ and 17extR - 5′ CGAGAAGAATATAAGCGGGAG 3′); and
6. 3′ UTR, FW4 DNA (3′endF - 5′ AGAAGCTTCGGTTTTTAATC 3′ and 3′endR - 5′ GGTCGCATCCGGCTATAAAC 3′).

The RNA probe templates corresponding to DNA probes 1–5, above, were amplified using the forward (F) primers above, but combined with reverse primers incorporating a T7 promoter:

1. *atp8* core—coreF and coreRT7—5′ T7AAGAAGTGCTATATGAGG 3′
2. *atp8*-1 extension—8-1F and 8-1RT7—5′ T7CTCTATTTTGAATTTTTTTCCG 3′
3. *atp8*-2 extension—8-2F and 8-2RT7—5′ T7CTCTATTTTTCATTTTTAGAATG 3′
4. FW2 *atp8* extension - 2extF and 2extRT7- 5′ CAGAAAGGGGAGGTCTGG 3′; and

5. FG17 *atp8* extension—17extF and 17extRT7—5′AGAAGAATATAAGCGGGAG 3′.

PCR, RT-PCR and 3′-RACE

PCR amplifications were performed using 20–100 ng of DNA template and Taq DNA polymerase (Fermentas): initial denaturation at 94°C, 4 min; 30 cycles of (94°C, 0.5 min; 50°C, 0.5 min; 72°C, 1 min), and final extension at 72°C, 10 min.

Contaminating DNAs in total RNAs used for RT-PCR or 3′-RACE were removed by treatment with the DNase I product DNA-*free* (Ambion). First-strand cDNA for RT-PCR was synthesized using random primers provided with the RETROscript™ kit (Ambion); RT-PCR was performed on 1 µl of the cDNA preparation, or RNA (no RT), using the conditions described above. First-strand cDNA for 3′-RACE was synthesized using a 1:1:1:1 mixture of 5′ GCGAGCACAGAATTAATACGT$_{12}$VN 3′, where N equals A, C, G or T; 3′-RACE was performed on 1 µl of the cDNA preparation, using a gene-specific forward primer and a primer corresponding to the anchor within the primer above (5′ GCGAGCACAGAATTAATACG 3′), under the conditions described above.

Amplified fragments were sequenced at a facility that uses an ABI Prism 377 DNA sequencer. Sequence alignment and analysis was with the program package BioEdit, version 5.0.9 (Hall 1999).

Antibody production

Antibodies against the synthetic peptide IRSKDPNSLEDILRK, which corresponds to amino acids 55–69 of the *atp8*-1 and *atp8*-2 reading frames, were raised in rabbits by standard methods. Peptide construction, immunization and serum collection were performed by Sigma-Genosys (Texas, USA).

Mitochondrial protein isolation

Mitochondria were isolated from immature carrot flowers by homogenization of tissue with a Polytron mixer, repeated differential centrifugations to remove nuclei and large cellular debris, and sucrose density gradient purification to remove chloroplasts, according to protocols outlined by Millar et al. (2001). After dilution with wash buffer, the gradient-purified mitochondrial band was pelleted first at 6,000 *g*, and the remaining supernatant was pelleted at 11,000 *g*. Each pellet was resuspended in 2× protein lysis buffer (2% SDS, 20% glycerol, 20 mM Tris (pH = 8), 2 mM EDTA, 160 mM DTT, 100 µg/ml PMSF), heated at 65°C for 10 min, and frozen.

Western immunodetection of ATP8

Aliquots of proteins from mt pellets collected at 6,000 *g* and 11,000 *g* after gradient-purification were electrophoresed in linear 12% SDS-polyacrylamide gels (Sambrook et al. 1989). The mt protein banding patterns were equivalent for the samples pelleted at 6,000 *g* and 11,000 *g*, but the samples from the11,000 *g* centrifugations were usually contaminated with lipids which severely disfigured protein banding below 25 kDa; samples from the 6,000 *g* centrifugations were used in subsequent western immunoblots. For accurate size determination, an unstained 10–200 kDa protein ladder (Fermentas) that is detectable with *Strep*-Tactin®-AP conjugate, was used as a molecular weight marker. Protein samples were electroblotted onto nitrocellulose and incubated with primary antiserum and secondary anti-rabbit antibody according to standard protocols (Sambrook et al. 1989); antiserum was diluted 1:400.

Results

Atp8 reading frames in carrot with and without C-terminal extensions

Atp8 reading frames with unique C-terminal extensions of 70 aa and 63 aa were PCR amplified from fertility-inducing mitotypes FW2 and FG17, respectively, using a reverse primer specific to the C-terminal extension of reading frame *atp8*-2 from sterility-inducing mitotype SW3 (Fig. 1). No products were obtained from FW2 or FG17 using a reverse primer specific to the C-terminal extension of *atp8*-1 from SW3, although the FW2 and FG17 *atp8* products were equivalent to *atp8*-1 in the core region (Supplementary Fig. 1); no products were obtained from mitotype FW4 using either primer. The C-terminal extensions of the *atp8* reading frames from SW3 (*atp8*-1 and *atp8*-2), FW2 and FG17 have regions of strong similarity at the nucleic acid level (Fig. 1a), but less similarity at the amino acid level (Fig. 1b) because most of the nucleic acid differences within the C-terminal extensions occur at non-silent positions.

Atp8 reading frames that terminated at a position homologous with those from other plant species were amplified from FW4 and FG17 via 3′-RACE (Fig. 2a). The 3′-RACE-amplified reading frames from FW4 and FG17 had 3′ UTRs of ~100 nt that were 98–100% identical and were polyadenylated within a 10 nt region (data not shown). PCR amplifications using primers derived from the 3′ UTRs confirmed that these normally-terminated reading frames were present in this form within the mtDNAs of FW4 and FG17 (data not shown); no products were amplified from cDNA or DNA templates of FW2 or SW3. The first 15 nt of the FW4 and FG17 *atp8* 3′ UTR sequences were very similar to genomically encoded *atp8* 3′ UTR sequences from three other plant species (Fig. 2a), but beyond this position, the FW4 and FG17 3′ UTRs align with

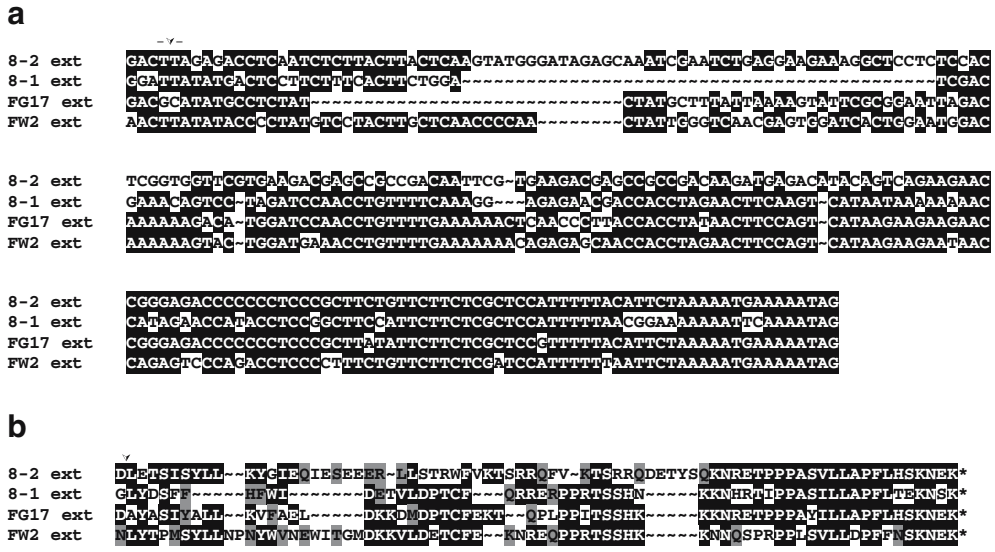

Fig. 1 The C-terminal extensions of *atp8* reading frames from different mitotypes of carrot. **a** An alignment of the nucleic acid sequences of the *atp8* C-terminal extensions from mitotypes SW3 (8-1 and 8-2), FG17 and FW2. A *half-shaded arrow head* indicates the position of the termination codon in homologous *atp8* reading frames from other plants. **b** An alignment of the amino acid translations of the *atp8* C-terminal extensions in (**a**). A *half-shaded arrow head* indicates the position of the termination codon in homologous *atp8* reading frames from other plants

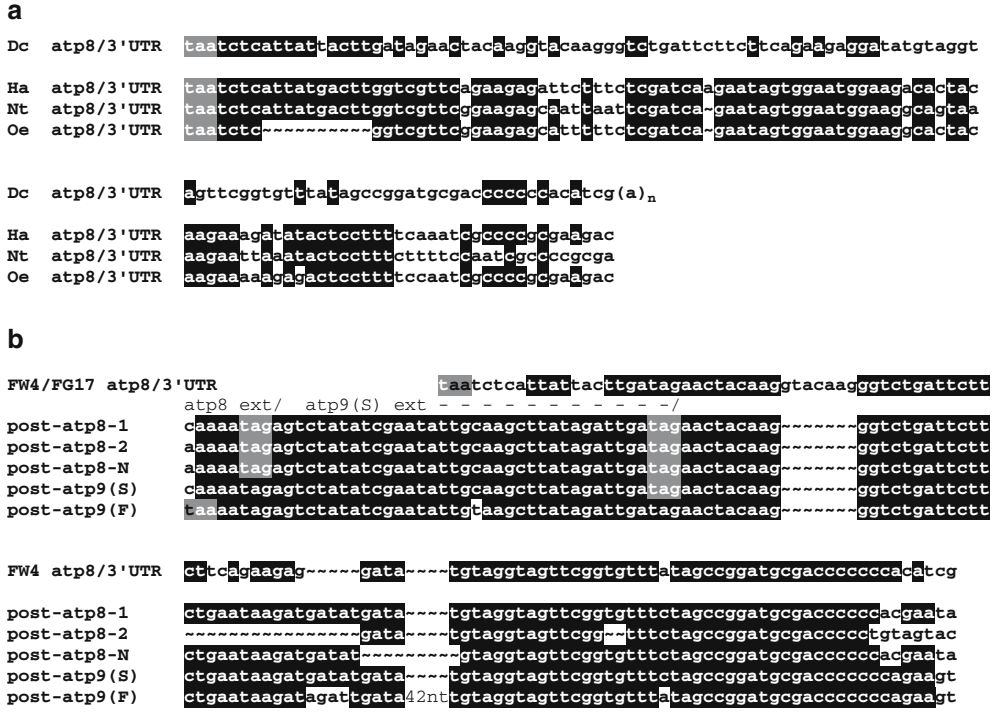

Fig. 2 The 3′ UTR of unextended *atp8* reading frames from carrot mitotypes FW4 and FG17. **a** The consensus 3′ UTR of the *atp8* reading frames amplified by 3′-RACE from carrot (Dc) mitotypes FW4 and FG17 aligned with *atp8* 3′ UTRs from *Helianthus annuus* (Ha; X57669), *Nicotiana tabacum* (Nt; NC_006581) and *Olea europaea* (Oe; AJ313433). The termination codons of the *atp8* reading frames are shaded in *gray*. **b** An alignment of the consensus *atp8* 3′ UTR from carrot mitotypes FW4 and FG17 with the sequences immediately downstream of: (1) the C-terminal extensions of *atp8* alleles *atp8*-1 (AY061991), *atp8*-2 (AY061992) from sterility-inducing mitotype SW3 of carrot, (2) the C-terminal extension of *atp8*-N (AY007818) from a fertility-inducing cytoplasm of carrot, (3) the C-terminal extension of *atp9* from a sterility-inducing cytoplasm of carrot (*atp9*(S); AJ009697 or AY007823), and (4) the *atp9* reading frame from a fertility-inducing cytoplasm of carrot (*atp9*(F); AJ009824 or AY007822). The last five nucleotides of the *atp8* C-terminal extensions and the complete *atp9*(S) C-terminal extension are included for comparison; the potential termination codons of each reading frame are shaded in *gray*

genomic sequences downstream of the *atp8* C-terminal extensions of *atp8*-1 and *atp8*-2 of SW3 (AY061991, AY061992), the C-terminal extension of the FW2-like sequence *atp8*-N (AY007818) and carrot *atp9* reading frames from sterility- and fertility-inducing cytoplasms (AJ009697 or AY007823, AJ009824 or AY007822; Szklarczyk et al. 2000) (Fig. 2b). Interestingly, the *atp8* 3′ UTR sequences from FW4 and FG17 terminated at the same point where the similarity shared by the sequences following the *atp8* extensions and the *atp9* reading frames also terminated (Fig. 2b).

Southern hybridization of mtDNAs from FW2, FW4 and FG17 with probes derived from the core region of *atp8* indicated that a single copy of *atp8* exists in each of these mitotypes (data not shown), although both extended and unextended versions of *atp8* were identified in FG17 using PCR-based methods. A probe specific to the FG17 extension did not hybridize to a Southern blot of FG17 DNA, suggesting that the predominant form of *atp8* in FG17 is the unextended version, and that the extended version is present in substoichiometric quantities and only detectable by PCR-based methods.

Transcription of *atp8* C-terminal extensions

Northern hybridization of total RNAs from flowers with a probe derived from the core region of *atp8* indicated that transcripts of *atp8* are present at similar levels in the flowers of all mitotypes, but that transcript size among the mitotypes ranges ~700–900 nt (Fig. 3a); additional faint transcripts are seen in mitotype SW3, the most predominant being ~1,600 nt long (Fig. 3a). Northern hybridization with probes specific to the various *atp8* C-terminal extensions from SW3, FW2 and FG17 indicated that the extensions from SW3 (*atp8*-1 and *atp8*-2) and FW2 are each present within the predominating *atp8* transcripts in those mitotypes (Fig. 3a); no transcripts corresponding to the FG17-type C-terminal extension were detected (data not shown). Additional small transcripts are detected with the *atp8*-1 extension-specific probe which implies that this extension may be post-transcriptionally processed, but corresponding small *atp8* transcripts from which the extension had been removed were not detected by the probe for the core region, even with long exposures. The *atp8*-2 extension-specific probe also detected several transcripts larger than the predominant primary transcript which suggests that transcripts of this *atp8* allele have multiple initiation or termination sites, or that sequences similar to this extension are present in transcripts from other parts of the genome (Fig. 3a).

Differently-sized ATP8 products from extended and unextended reading frames

Polyclonal antibodies were raised in two different rabbits against a peptide sequence from within the core region of the *atp8* reading frame (IRSKDPNSLEDILRK) and used as probes in Western immunoblots of mitochondrial proteins from flowers of mitotypes SW3, FW4 and FG17; insufficient material for mitochondrial isolations was available from FW2. The predicted sizes of products from the different mitotypes were as follows: (1) FW4, 18 kDa because of the lack of a C-terminal extension; (2) SW3, 25 kDa

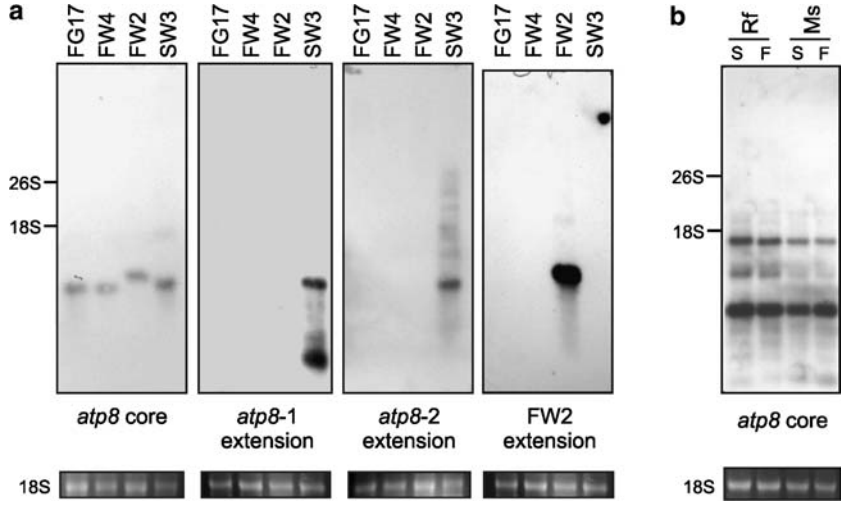

Fig. 3 Transcript analysis of the conserved core and C-terminal extensions of *atp8* in carrot. **a** Northern hybridizations of probes from the core region of *atp8* and the C-terminal extensions from *atp8*-1, *atp8*-2 and FW2 (see Materials and methods) to total RNAs from mitotypes FG17, FW4, FW2 and SW3 of carrot. Each probe was hybridized to independent blots of the same RNA; ethidium bromide-stained 18S rRNAs are shown as loading controls. The positions of the 26S and 18S rRNAs are indicated. **b** Northern hybridization of the probe from the core region of *atp8* to total RNAs from flowers of mitotype SW3 that were phenotypically sterile (*S*) or fertile (*F*) as a result of segregation for alleles at either Restorer-of-fertility (*Rf*) or Maintainer-of-sterility (*Ms*) nuclear loci. Ethidium bromide-stained 18S rRNAs are shown as loading controls; the positions of the 26S and 18S rRNAs are indicated

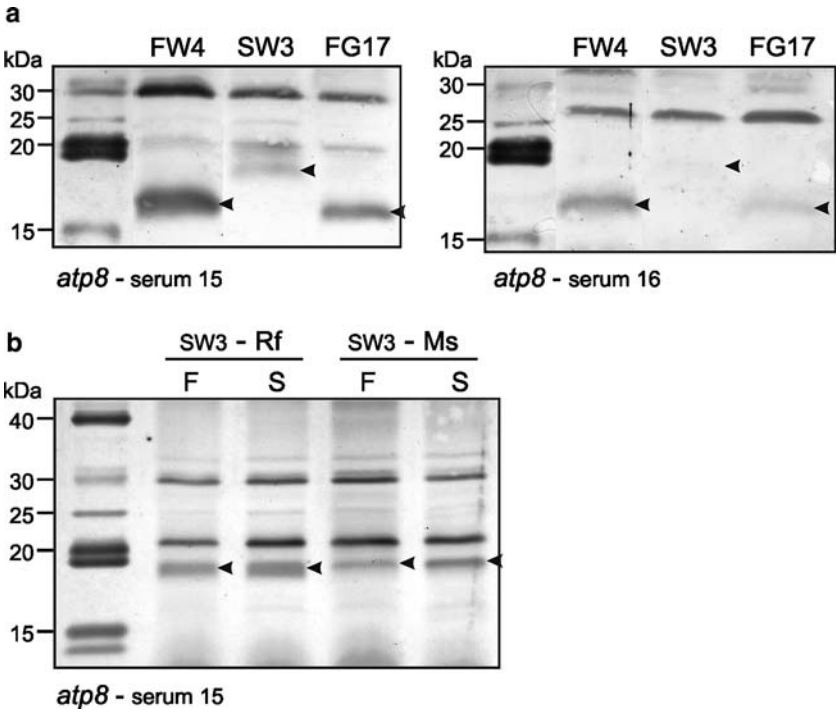

Fig. 4 Western analysis of ATP8 products in carrot. **a** Mitochondrial proteins from flowers of mitotypes FW4, SW3 and FG17 probed with sera from two different rabbits (serum 15, serum 16) that were immunized against a peptide sequence within the *atp8* core region (see Materials and methods). *Arrowheads* indicate the *atp8* peptide-specific bands; molecular marker sizes are at *left*. **b** Mitochondrial proteins from flowers of mitotype SW3 that were phenotypically fertile (*F*) or sterile (*S*) as a result of segregation for alleles at either Rf or Ms nuclear loci, probed with serum from rabbit 15 immunized against a peptide sequence within the *atp8* core region. *Arrowheads* indicate the peptide-specific bands; molecular marker sizes are at *left*

and/or 27 kDa because of C-terminal extensions encoded by *atp8*-1 and *atp8*-2, respectively; and (3) FG17, 18 kDa and/or 25 kDa because of the absence or presence, respectively, of a C-terminal extension. Both sera produced background bands due to non-specific binding, but the non-specific banding patterns for each serum were unique and relatively faint in the region of expected ATP8 binding (15–28 kDa), and so did not significantly interfere with visualization of the ATP8 product. Bands of ~17 kDa, nearly identical to the predicted size of 18 kDa, were detected in mitochondria from FW4 and FG17 by both sera (Fig. 4a). A unique band of ~19 kDa was detected in mitochondria from SW3 by both sera (Fig. 4a and data not shown), which is a size significantly smaller than the 25–27 kDa predicted by translation of the extended reading frame; a faint band at 25 kDa detected by serum 15 must be attributed to non-specific binding because of its presence in all mitotypes (Fig. 4a).

Unaltered *atp8* transcripts and protein products in petaloid and fertility-restored flowers

Atp8 transcripts and proteins were compared from flowers of mitotype SW3 that were either phenotypically fertile or sterile as a result of segregation for alleles at either Ms or Rf nuclear loci. There was no difference in size or abundance of either *atp8* transcripts (Fig. 3b) or ATP8 protein products (Fig. 4b) from segregating sterile and fertile flowers of the SW3 mitotype.

Discussion

A diverse number of mt reading frames from many different plant species are extended at the C-terminal end because of the absence of a stop codon at the expected position (Bailey-Serres et al. 1986; Saalaoui et al. 1990; Kaleikau et al. 1992; Dong et al. 1998; Ducos et al. 2001; Perrotta et al. 2002; Robison and Wolyn 2006), but it is not clear how such extensions originate and what their role(s) may be in the final gene product. The similarity of the four unique *atp8* C-terminal extensions from carrot indicates that these extensions share the same sequence origin. However, non-functionality is indicated because: (1) the level of similarity at the amino acid level is much lower than the level of similarity at the nucleic acid level, and (2) the level of sequence conservation within the extensions is much less than that within the core *atp8* region or the adjoining downstream 3′ UTR region. All of these observations are consistent with the creation of the extensions by a single recombination event in an ancestral carrot mitochondrial genome, prior to mitochondrial divergence.

The detection of a 19 kDa product in mitotype SW3 which is larger than the 17 kDa products from unextended *atp8* reading frames but smaller than the predicted 25–27 kDa products, indicates that at least a small portion of the C-terminal extension may be present within the mature protein. The COX1 product from an extended reading frame in Sorghum has also been found to be smaller than predicted for complete translation of the extension, but larger than the product from unextended reading frames (Bailey-Serres et al. 1986). Putting our data together with those of Nakajima et al. (2001), wherein an extension-specific antibody detected a 25 kDa product, we propose that the complete extension may be initially translated, but then the protein is quickly processed to a final size of 19 kDa. The *rps2* reading frame of maize encodes a C-terminal extension that appears to be initially translated because it can be detected among *in organello*-synthesized RPS2 proteins, but the extension must then be post-translationally removed because the mature protein within the ribosome is the size expected from an unextended reading frame (Perrotta et al. 2002).

The presence of *atp8* reading frames with C-terminal extensions in both sterility- and fertility-inducing mitotypes indicates that *atp8* C-terminal extensions per se cannot be correlated with cms in carrot, and the absence of an unextended version of *atp8* in a fertility-inducing mitotype (FW2), even at substoichiometric levels (data not shown), indicates that the encoding of unextended *atp8* reading frames does not determine fertility. However, it has been contended that the encoding of an *atp8* reading frame with a particular C-terminal extension correlates with petaloidy in carrot (Nakajima et al. 2001). The best evidence that a molecular determinant of male sterility has been correctly identified is the loss or alteration of that determinant, or its product, by the action of nuclear restorer-of-fertility genes; restoration to phenotypically-fertile flowers should correlate with a change in the product of the proposed cytoplasmic determinant. There is presently no evidence to support a role for the *atp8* gene(s) of carrot in petaloid-type cms because there is no change in the quantity or size of transcripts or translated products from these reading frames in flowers that have been restored to fertility under the action of nuclear Ms or Rf alleles. A mitochondrial factor positively correlated with petaloidy in carrot presently remains unreported.

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