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ORIGINAL PAPER

Cytoplasmic suppression of Ogura cytoplasmic male sterility in European natural populations of *Raphanus raphanistrum*

Sandra Giancola · Yong Rao · Sophie Chaillou · Sophie Hiard · Alfred Martin-Canadell · Georges Pelletier · Françoise Budar

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Abstract The Ogura cytoplasmic male sterility (CMS) of radish has been used for hybrid seed production in radish and *Brassica* crops. It is the only CMS system occurring in wild populations for which the gene responsible for sterility and a restorer gene have been formally identified. In Japan, gynodioecious populations of radish carrying Ogura or an Ogura-related cytoplasm have been described. The occurrence of restorer genes for the Ogura CMS in wild radish (*Raphanus raphanistrum*) in France led us to search for the corresponding male sterility gene (*orf138*) in several natural populations in France, England and Lebanon. We detected the *orf138* gene, by PCR, at low frequency, in three populations from France and one from Southern England. Further molecular characterization showed that these plants carried a cytoplasm closely related to the original Ogura cytoplasm, with a variant *orf138* coding sequence, previously reported to be ancestral. We performed crosses with sterile and maintainer radish lines, to test the ability of this wild Ogura-related cytoplasm to induce sterility. Surprisingly, the European Ogura-related cytoplasm did not cause sterility. Northern blots and circular RT-PCR analyses showed that *orf138* gene expression was impaired in these plants because of a novel cytoplasm-dependent transcript-processing site.

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Sandra Giancola and Yong Rao contributed equally to this work.

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S. Giancola · Y. Rao · S. Chaillou · S. Hiard · A. Martin-Canadell · G. Pelletier · F. Budar (✉)
Station de Génétique et d'Amélioration des Plantes, Institut Jean-Pierre Bourgin, INRA UR254, Versailles, France
e-mail: budar@versailles.inra.fr

Present Address:
Y. Rao
Institute of Oil Crops, Guizhou Academy of Agricultural Sciences, Xiaohe District, Guiyang, Guizhou Province, People's Republic of China

Introduction

Cytoplasmic male sterility (CMS) systems determine the reproductive biology of plants through the interaction of a male sterility-inducing cytoplasm and nuclear restorer genes. They have been widely used for the production of hybrid crop seeds (Havey 2004). CMS systems have attracted considerable attention in two different scientific communities. Population geneticists have studied CMS in natural populations, in which it is expressed as gynodioecy. Gynodioecy is defined as the co-occurrence of hermaphrodites and females in a population or species (Darwin 1877). The studied cases of gynodioecy result from naturally occurring CMS systems. Population geneticists have studied principally the maintenance of genetic and phenotypic polymorphisms, and their mode of evolution (Charlesworth 2002; Bailey et al. 2003; Saur Jacobs and Wade 2003). According to the most widely accepted models, nuclear restorer genes are selected in the presence of the sterility-inducing cytoplasm because, by allowing the production of pollen, they improve their own transmission to the progeny. Conversely, if the sterility-inducing

cytoplasm is not present, they are obviously not selected, and may even be disadvantaged. This situation, called "cost of restoration", was predicted by recent models (Bailey et al. 2003) and leads to the selection against restorers in the absence of the sterility-inducing cytoplasm. Most, if not all, of the studied examples of gynodioecy in natural populations have involved CMS systems with unknown genetic determinants. Our understanding of the molecular evolution of these systems therefore remains largely theoretical. Molecular geneticists have tried to identify the genes involved, mainly in crops or crop-related species, in which such systems can be exploited for hybrid seed production. A number of mitochondrial genes have been correlated with, or formally implicated in male sterility (for reviews, Hanson and Bentolila 2004; Budar et al. 2006). Nuclear genes for the restoration of fertility (*Rf*) have been identified (Cui et al. 1996; Bentolila et al. 2002). All except the maize Texas-CMS restorer *Rf2* encode proteins of the PPR family, involved in the posttranscriptional control of mitochondrial and chloroplast gene expression (Lurin et al. 2004). The assumed molecular function of PPR proteins is consistent with the general observation that fertility is restored by impairing sterility gene expression through posttranscriptional mechanisms affecting sterility gene mRNA maturation, editing, or accumulation (Wang et al. 2006). The structure of the first identified *Rf* loci in *Petunia*, radish and rice led to comparisons with disease resistance loci, as selection for *Rf* loci occurs in response to the establishment of a sterility-inducing cytoplasm in a population (Touzet and Budar 2004).

The Ogura CMS system is the only naturally occurring CMS system in which the genes responsible for sterility and restoration have been formally identified. The Ogura CMS, originally identified in a Japanese radish cultivar (Ogura 1968), was introduced into *Brassica* crops for hybrid seed production (Budar et al. 2004). The mitochondrial gene responsible for Ogura male sterility, *orf138*, was shown to be constitutively expressed in sterile plants (Bonhomme et al. 1991, 1992; Krishnasamy and Makaroff 1993; Grelon et al. 1994). In the original Ogura cytoplasm and in male sterile *Brassica* cybrids, the *orf138* gene is expressed as a bicistronic transcript, together with the *orfB* gene (Bonhomme et al. 1992; Krishnasamy and Makaroff 1993). We have shown, in *Brassica* cybrids, that the coexpression of *orf138* with *orfB* is involved in stabilization of the bicistronic transcript and expression of the sterility trait (Bellaoui et al. 1997). The Ogura CMS has been detected in wild and cultivated Asian *Raphanus* genotypes (Yamagishi and Terachi 1994a, b, 1996, 1997). It is present in natural gynodioecious populations of wild radish in Japan (Murayama et al. 2004). An analysis of the *orf138* gene sequence of various cytoplasms from Asian wild and cultivated radishes led to the identification of several intragenic variations in the coding sequence of the gene, making it possible to infer phylogenic relationships (Yamagishi and Terachi 2001). A nuclear locus restoring fertility for Ogura CMS was recently identified and described in radish (Brown et al. 2003; Desloire et al. 2003; Koizuka et al. 2003).

Restorers for the Ogura CMS were discovered in natural French populations of *Raphanus raphanistrum* after interspecific crosses between *Brassica napus* male-sterile plants and wild radish (Eber et al. 1994). We report here the identification of a wild radish cytoplasm carrying the *orf138* gene in European populations that also carry restorer genes for the Ogura CMS. We show that this cytoplasm cannot induce sterility, despite its strong similarity to the original Ogura cytoplasm. We also present an analysis of the expression of the sterility gene in this newly identified cytoplasm. Our results demonstrate the existence of an unprecedented and unexpected situation within a CMS system.

Materials and methods

Plant materials

The cultivated radish (*Raphanus sativus*) maintainer genotype used as the reference genotype was the "L7" line created by Bonnet (1977). It is available as fertile plants with normal radish cytoplasm (L7F plants) or as male sterile plants with the Ogura cytoplasm (L7S plants). Plants were grown and crosses were performed in the greenhouse.

Wild radish (*Raphanus raphanistrum*) plants were collected from natural populations (see Table 1), either as leaf samples, or as seeds of maternal descent. The leaf samples were dried in an oven (50–60°C) and stored at room temperature before genomic DNA extraction. Seeds were sown in the greenhouse and fresh leaf material was collected from one individual per mother plant for genomic DNA extraction.

Isolation of nucleic acids

Genomic DNA for PCR analysis was extracted in 96-well plates, using a modified version of a previously described procedure (Loudet et al. 2002). Fresh or oven-dried tissues and a 4 mm glass bead were placed in tubes held in a 96-well polypropylene storage plate. The

Table 1 Names and locations of the natural populations studied

Population name	Location (country)	Materials collected
POP2	Rennes (France)	Seeds
POP9	Boullay-les-Troux (France)	Seeds
POP12A	Poilly-lez-Gien (France)	Seeds
POP13	Saint-Martin/Ocre (France)	Seeds
POP14	Pont-à-Marcq (France)	Seeds
POPN199	Montreuil-sur-mer (France)	Seeds
AZB	Azay-le-Rideau (France)	Seeds
CR	Le Croisic (France)	Seeds
SG	Saint Gildas (France)	Leaves
POPE	Etacq, Jersey (Channel Islands)	Seeds
CHOA	Chausey (Channel Islands)	Leaves
RB	Rocquaine Bay, Guernsey (Channel Islands)	Seeds
LIZ	Lizard Point (England)	Leaves
LBA	Bater ech Chouf (Lebanon)	Leaves
LBB	Marj el zhour (Lebanon)	Leaves
LBC	Dahr el Souan (Lebanon)	Leaves
LBD	Qartaba (Lebanon)	Leaves

tissues were frozen in liquid nitrogen and ground with an MM300 vibrator (Retsch GmbH). Extraction buffer (200 mM tris–HCl pH7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS, 1.7 mg/ml proteinase K) was added (200 μl per tube) to the ground tissue and the mixture was incubated for 20 min at room temperature. After centrifugation for 15 min at 3,000*g*, 100 μl of supernatant was added to 100 μl of isopropanol in one of the wells of a 96-well plate. The plate was incubated for 10 min at room temperature, the DNA was pelleted by centrifugation for 15 min at 3,000*g*, and the supernatant was discarded. The pellet was air-dried and the DNA resuspended in 50–100 μl of water. Typically, we used 2 μl of the DNA solution for PCR. The quantity of DNA solution used was adjusted when the DNA yield was insufficient (especially for oven-dried samples).

Genomic DNA was extracted for DNA hybridization analysis as previously described (Dellaporta et al. 1983), using fresh samples harvested in the greenhouse. It was not possible to obtain genomic DNA suitable for hybridization analysis from dried leaf samples.

Total RNA was extracted by grinding fresh samples harvested in the greenhouse in liquid nitrogen and using Trizol reagent (Invitrogen) according to the manufacturer's instructions. It was not possible to obtain RNA from dried leaf samples.

Amplification analyses

Most amplification reactions were performed in 25 μl of reaction mixture containing 75 mM tris–HCl pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% tween, 2.5 mM $MgSO_4$, 0.3 mM of each dNTP, 0.1 μM of each primer, 1–2 units of *Taq* DNA polymerase prepared in the laboratory. Typical cycling reactions consisted of 1–5 min at 95°C followed by 25–30 cycles [30 s at 94°C, 30 s at annealing temperature (see Table 2), 1–3 min at 72°C (depending on the length of expected amplification product)] and a final 10 min at 72°C. Amplification products were analyzed by 1% agarose gel electrophoresis.

Sequencing analyses

One region of the mitochondrial genome, corresponding to the *orf138* locus, and two regions of the plastid genome, corresponding to the intron of the K-tRNA gene and the intergenic region between the L- and F-tRNA genes (Grivet et al. 2001), were sequenced in several individuals (see "Results"). All sequencing was carried out by Genoscreen. The *orf138* locus was directly sequenced from the product amplified with primers orf138-F2 and orfB-R1 (Table 2), using the same primers. The plastid genome regions were amplified with the primers K1-M13F and MatK1-M13R, and with the primers trnL-M13F and trnF-M13R. Amplification products were then directly sequenced with M13F and M13R primers (Table 2).

RNA and DNA hybridization analyses

Total DNA was digested with the chosen restriction enzymes, in the buffer recommended by the enzyme supplier (Fermentas), supplemented with 4 mM spermidine. The digestion products were subjected to electrophoresis in 0.6 or 0.8% (depending on the size of the expected hybrizing fragments, see figure legend) agarose gels in TBE buffer (Ausubel et al. 1990). The bands were then transferred to a nylon membrane (Genescreen), according to the manufacturer's instructions.

Total RNA was loaded onto a 1.2% agarose gel containing 8% formaldehyde in 0.5× MOPS buffer (Ausubel et al. 1990). Electrophoresis was conducted in 1× MOPS buffer at 50V overnight (ca. 18 h). The bands were then transferred to a nylon membrane (Genescreen), according to the manufacturer's instructions.

The fragments used as probes were obtained by amplification from plant total DNA or from cloned fragments of mitochondrial DNA. Their positions on the *orf138* locus are shown in Fig. 1 (hatched boxes). Probe A (*orf138*) was obtained by PCR with primers orf138-F1 and orf138-R on sterile plant DNA; probe B (*orfB*) was obtained by PCR with primers orfB-F and orfB-R2 on plant DNA; probe C (*fMtRNA*) was obtained by PCR with primers TRNAFM-F and TRNAFM-R on plant DNA; probe D (5′ region of

Table 2 Primers used in this study

Primer	5′–3′sequence	Annealing temperature for PCR(°C)	Amplicon size(bp)
cobU cobL	TCTTCTCTCGGGGTCATCCT CCCCCTTCAACATCTCTCAT	53	700
orf138-F1	GCATCACTCTCCCTGTCGTTATCG		512
orf138-R	ATTATTTTCTCGGTCCATTTTCCA		
orf138-F2 orfB-R1	GAAACGGGAAGTGACAATAC GTACTCCATCTCCATCATTGC	51	788
cDNA-priming cRT-F cRT-R	TGGGGTCCTTGCTCTGGATGGTCT GCTCTAGAGACTTATTGGGAAAAAGGAGG GCATTATTTTCTCGGTCCAT	52	variable
orfB-F orfB-R2	TCAACAACCAACCACAACTTT TACAAGTGATCCACCTTCCAG	52°C	520
TRNAFM-F TRNAFM-R	ACGTGTAGCCCTGTATGGACT GGTATTGTCACTTCCCGTTTC	54	398
UF UR	GTAAAACGACGGCCAGT GGAAACAGCTATGACCATG	53	variable
K1-M3F MatK1-M13R	CACGACGTTGTAAAACGACGTTGCCCGGGATTCGAA GGATAACAATTTCACACAGGATTAGGGCATCCCATTAGTA	50	706
trnL-M13F trnF-M13R	CACGACGTTGTAAAACGACGGTTCAAGTCCCTCTATCCC GGATAACAATTTCACACAGGATTTGAACTGGTGACACGAG	52	450
M13F M13R	CACGACGTTGTAAAACGAC GGATAACAATTTCACACAGG		

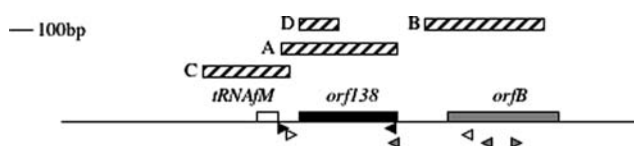


Fig. 1 The *orf138* locus. The *orf138* locus is presented as described for the original Ogura cytoplasm (Bonhomme et al. 1992; Krishnasamy and Makaroff 1993; Bellaoui et al. 1997). The *boxes* on the horizontal line represent the coding sequences of genes: *white box*, gene for the formylmethionine tRNA (*tRNAfM*); *black box*, *orf138*; *gray box*, *orfB* (or *atp8*). *Arrowheads* below the line indicate the positions of the oligonucleotide primers used in this study (see also Table 2): *black arrowheads*, primers for PCR detection of the *orf138* gene in wild plants (orf138-F1 & orf138-R); *white arrowheads* primers for amplification and sequencing of *orf138* in wild plants (orf138-F2 & orfB-R1); *gray arrowheads* primers for cRT-PCR, the internal primer was used for cDNA priming (cDNA priming), the external primers for amplification (cRT-F & cRT-R). The *hatched boxes* above indicate the regions used as probes in the DNA and RNA hybridization experiments shown in Figs. 2 and 3

orf138, 390 bp) was obtained by PCR with primers UF and UR ("universal" primers for pBluescript) on a plasmid derived from pBluescript carrying the first 57 codons of *orf138* (Duroc et al. 2005). When necessary, the amplification products were purified after agarose electrophoresis, before labeling with a random priming kit (Promega) and α^{32}P dCTP, according to the manufacturer's instructions.

Hybridizations were carried out as previously described (Ausubel et al. 1990).

Circular RT-PCR (cRT-PCR) analyses (Kuhn and Binder 2002)

Circular RT-PCR was performed as described elsewhere (Perrin et al. 2004), using the primers described in Table 2. Their positions on the *orf138* locus are given in Figs. 1 and 4. Cycling reactions were carried out as follows: 5 min at 95°C, followed by 10 cycles (30 s at 95°C, 45 s at 62°C minus 1°C each cycle, 1.5 min at 72°C), 25 cycles (30 s at 95°C, 45 s at 52°C, 1.5 min at 72°C), and a final 10 min at 72°C. Amplicons were inserted into pTOPO (Invitrogen), and the sizes of the inserts were estimated by PCR amplification, using UF and UR primers and agarose gel electrophoresis. Genome Express sequenced certain selected clones, using the UF and UR primers.

Results

The *orf138* gene is present at low frequency in non-gynodioecious European wild populations of *Raphanus raphanistrum*

The known occurrence of fertility restorers for Ogura CMS in natural populations of *Raphanus raphanistrum* (wild radish) in France (Eber et al. 1994) led us to search for the Ogura CMS mitochondrial gene in

populations of wild radish from Europe and the Middle East. We screened a total of 17 populations of *R. raphanistrum*, from various locations in France, the Channel Islands, England, and Lebanon (Table 1). None of the populations studied was considered gynodioecious on the basis of in situ observations. We amplified the mitochondrial *cob* gene and *orf138* amplification in a single reaction, to distinguish between failed amplification due to poor quality DNA and failed amplification due to the absence of the *orf138* gene sequence. Only samples giving a *cob* amplification product and no *orf138* amplification product were considered negative for the *orf138* gene. The *orf138* gene was detected in three populations from France and one population from southern England, at a low frequency (see Table 3). We investigated whether *orf138* was associated with *orfB* in these cytoplasms, as in the Ogura cytoplasm, by amplification with the orf138-F2 and orfB-R1 primers (see Fig. 1). In all cases, the *orf138* gene present in these European wild radishes was associated with *orfB*, as in the Ogura cytoplasm. The 788 bp region of the *orf138* locus between the orf138-F2 and orfB-R1 primers was sequenced for two individuals in each population (with the exception of the LIZ population, in which only one positive individual was detected, and sequenced). All the sequences were identical and corresponded to the B-type sequence identified as the probable ancestral sequence for the *orf138* gene: they carried the previously described silent A99G substitution in the *orf138* coding region (Yamagishi and Terachi 2001). The cytoplasm detected in European wild radishes is therefore not identical to the Ogura cytoplasm and is hereafter referred to as the Ogura-related cytoplasm.

Table 3 Detection of *orf138* in natural populations of *Raphanus raphanistrum*

Population	Year of sampling	Number of tested individuals[a]	Number of individuals with *orf138*[b]
POP2	1998	223[c]	15
	1999	83	0
	2001	150	2
POP9	1999	66	0
POP12A	1999	43	0
POP13	1999	70	0
POP14	1999	62	0
POPN199	1999	55	8
AZB	2000	15	0
CR	2004	24	0
SG	2004	23	2
	2005	17	1
POPE	2000	24	0
CHOA	2004	46	0
RB	2000	12	0
LIZ	2003	56	1
LBA	2005	42	0
LBB	2005	36	0
LBC	2005	40	0
LBD	2005	30	0

[a] Only samples giving amplification with the *cob* primer pair were considered

[b] Individuals giving positive amplification with the *orf138* primer pair

[c] Seeds were harvested in bulk from the natural population this particular year, therefore, different individuals tested may have originated from the same female parent

The Ogura-related cytoplasm of European wild radish does not induce male sterility

The B-type sequence of *orf138* described by Yamagishi and Terachi (2001) induces male sterility in Japanese wild radishes (Yamagishi and Terachi 1994a, 1997). At least one of the European populations with individuals carrying the Ogura-related cytoplasm contains restorers of fertility (Eber et al. 1994). Thus, provided that fertility restorers are segregated out, we would expect to observe male sterility caused by the Ogura-related cytoplasm in the progeny of wild mother plants. We crossed individuals carrying the Ogura-related cytoplasm from the initially prospected populations (POP2 and POPN199) with radish tester genotypes. The same plants (grown in the greenhouse from seeds harvested from natural populations, maternal descent) were used in both crosses. We first used the wild plants as male parents for the pollination of male-sterile radish plants (L7S). In the progenies of these crosses, we obtained male fertile (restored) and male sterile plants. The small numbers of seeds collected from the crosses precluded a precise genetic analysis of restoration. However, among the crosses performed with 13 different wild plants used as male parents, and which gave between one and nine seeds, five gave at least one sterile progeny, showing that restoration was not fixed in the population considered. We also used the wild plants as female parents and pollinated their emasculated flowers with pollen from the maintainer radish line (L7F). Based on the results obtained for the first cross, we sowed only seeds obtained from plants giving male sterile progeny when used as paternal parents—i.e., those not homozygous for fertility restorers. Similar numbers of seeds were obtained from both crosses (between 3 and 11 seeds). No male sterile plant was observed in the maternal progeny of wild plants, in contrast to what would be expected if these plants carried a sterility-inducing cytoplasm. The cross was repeated with one of the maternal plants and 30 new progenies were all fertile. Several plants from three backcross progenies were backcrossed again with the radish maintainer line

(L7F) and gave between 10 and 50 BC2 progenies. Once again, no male sterile plant was obtained, demonstrating that despite the apparently normal male sterility gene sequence, the cytoplasm of wild plants could not induce male sterility.

The European Ogura-related cytoplasm is very similar to the Ogura cytoplasm

The inability of the Ogura-related cytoplasm to cause sterility may be linked to a rearrangement of mitochondrial DNA at the *orf138* locus undetectable by PCR. We therefore carried out DNA hybridization analyses on several plants from the POP2 and POPN199 populations, using probes detecting the *orf138* coding sequence, the *orfB* gene, and the *fMet-tRNA* gene. Wild plants carrying the Ogura-related cytoplasm gave similar hybridizing fragments to the Ogura cytoplasm (Bonhomme et al. 1991, 1999). The *orf138* probe detected the Ogura-specific *Nco*I 2.5 kb and *Bgl*I 9 kb fragments. The *orfB* probe also detected only these fragments, demonstrating that the only *orfB* gene present in the Ogura-related cytoplasm is linked to *orf138*. The *fMet-tRNA* probe detected the same fragments as the *orf138* probe plus another fragment (approximately 18.5 kb for the *Bgl*I digestion), showing that this gene is duplicated in the Ogura-related cytoplasm, as in the original Ogura cytoplasm (Krishnasamy and Makaroff 1993). Furthermore, none of the wild plants testing negative for *orf138* in specific PCR assays had hybridization fragments in common with the Ogura or Ogura-related plants. Moreover, their hybridization profiles differed (see for example samples 4 and 5 in Fig. 2b, and samples 8 and 9 in Fig. 2c), showing that the populations studied contained various cytoplasms lacking *orf138*. We also sequenced two small regions of the plastid genome (see "Materials and methods") from several individuals of each prospected population. For both regions, the sequences obtained from wild plants carrying the Ogura-related cytoplasm were identical to those from plants with the Ogura cytoplasm, and different from those of wild plants lacking the *orf138* gene (see Electronic supplementary data). These analyses confirm that different cytoplasms lacking *orf138* coexist in the wild populations.

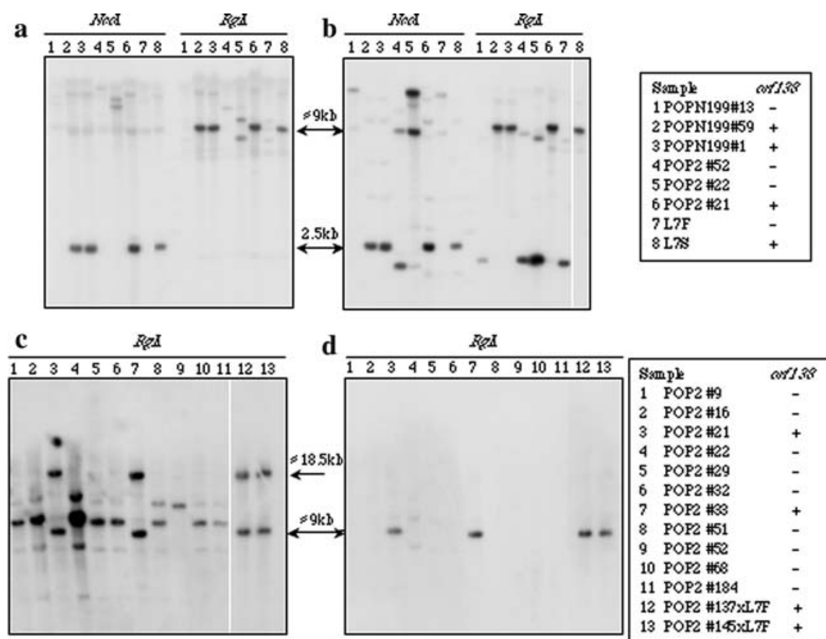
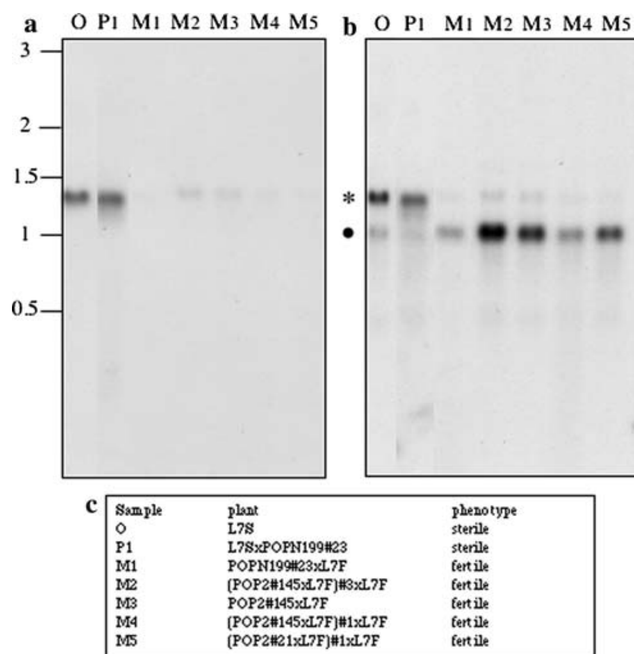


Fig. 2 DNA hybridization analysis of wild plants. Two successive hybridizations of the same membrane are shown in **a** and **b**. The same is true for **c** and **d**. The loaded samples are indicated for each gel in the boxes on the *right*. POPX#n means "plant number n in population POPX". L7S is sterile and carries the Ogura cytoplasm, L7F is the fertile maintainer (see "Materials and methods"). POPX#n xL7F means that the DNA was extracted from the maternal progeny of the wild plant, obtained by pollination with L7F. The signs in the column furthest to the *right* indicate whether the considered plant did (+) or did not (−) generate an amplification product in the *orf138*-specific PCR. **a**, **b**: *Nco*I and *Bgl*I digestions of total DNA were separated by electrophoresis in a 0.8% agarose gel and blotting onto a membrane. The membrane was (**a**) hybridized with an *orf138* probe (*hatched box A* in Fig. 1), stripped and (**b**) reprobed with an *orfB* probe (*hatched box B* in Fig. 1). **c**, **d** *Bgl*I digestions of total DNA were separated by electrophoresis in a 0.6% agarose gel and blotting onto a membrane. The membrane was (**c**) hybridized with a *tRNAfM* probe (*hatched box C* in Fig. 1), stripped, and (**d**) reprobed with an *orf138* probe (*hatched box A* in Fig. 1)

The expression of *orf138* is impaired in the European Ogura-related wild cytoplasm

We analyzed *orf138* expression in the wild Ogura-related cytoplasm, carrying out RNA hybridization analyses to determine the reasons for the lack of sterility in maternal descent from wild mother plants. These analyses were performed on plants with the European Ogura-related cytoplasm, which are fertile, and sterile plants with the Ogura cytoplasm, using *orf138* and *orfB* probes. The *orf138-orfB* cotranscript accumulated as the major RNA in plants with the Ogura cytoplasm (Fig. 3), as previously shown (Bonhomme et al. 1992). However, this cotranscript was present in extremely small amounts in plants with the wild Ogura-related cytoplasm, despite these plants being produced by one (M1, M3) or two (M2, M4, M5) back-crosses with the L7F maintainer radish line. A shorter RNA, not detected with the probe covering the 5′ part of *orf138*, accumulated as the major *orfB* RNA in these plants. As this cytoplasm contains no other *orfB* gene (Fig. 2) the *orfB* mRNA must originate from the *orf138-orfB* locus.

We defined the *orfB* mRNA ends by circular RT-PCR (cRT-PCR) analysis (Kuhn and Binder 2002) in several male fertile plants with the wild Ogura-related cytoplasm (Fig. 4). Briefly, RNA molecules are circularized by T4 RNA ligase before first strand cDNA synthesis followed by PCR with primers directed outwards the gene, thus amplifying in one step both 5′ and 3′ ends of the RNA. As a control, the same experiment was performed on male sterile plants with the Ogura cytoplasm. The sizes of the major cRT-PCR products obtained were consistent with the major *orfB* mRNA detected in RNA hybridization analysis (Fig. 4a). After cloning, however, inserts of different



Sample	plant	phenotype
O	L7S	sterile
P1	L7SxPOPN199#23	sterile
M1	POPN199#23xL7F	fertile
M2	(POP2#145xL7F)#3xL7F	fertile
M3	POP2#145xL7F	fertile
M4	(POP2#145xL7F)#1xL7F	fertile
M5	(POP2#21xL7F)#1xL7F	fertile

Fig. 3 RNA hybridization analysis. **a**, **b** The same membrane was hybridized (**a**) with a probe specific for the 5′ part of the *orf138* gene (*hatched box D* in Fig. 1), stripped and then reprobed (**b**) with a probe specific for the *orfB* gene (*hatched box B* in Fig. 1). Figures on the left indicate the positions of size markers given in kb. **c** Description of the samples. The plants are designated as in Fig. 2. (POPX#nxL7F)#mxL7F is the progeny of the second back-cross with L7F, performed on plant #m of the first backcross of plant #n from POPX by L7F. P1 and M1 were obtained from the same wild plant, crossed either as a male parent with the L7S plant, giving P1, or as female parent with the L7F plant, giving M1. P1 therefore has the Ogura cytoplasm and is male sterile, whereas M1 has the wild Ogura-related cytoplasm and is male fertile. The *asterisk* indicates the position of the major RNA in plants carrying the original Ogura cytoplasm (*O*, *P1*); the *black dot* indicates the position of the major RNA in plants with the wild Ogura-related cytoplasm

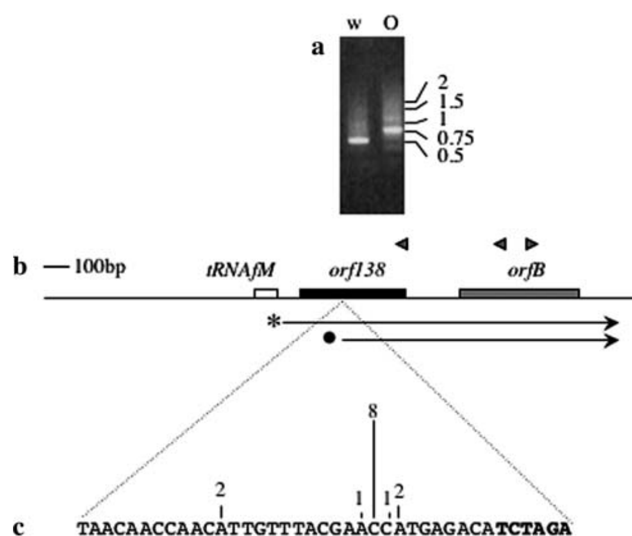


Fig. 4 cRT-PCR analysis. **a** Typical cRT-PCR result. Amplification products, obtained as described in the "Materials and methods", were separated by electrophoresis in a 1% agarose gel. *w* products obtained with RNA from a plant with the wild Ogura-related cytoplasm (in this case POPN199#23xL7F). *O* products obtained with RNA from a plant with the Ogura cytoplasm (in this case L7Sx POPN199#23). **b** Representation of the *orf138-orfB* locus (see also legend of Fig. 1). The *horizontal arrows* represent the RNA molecules mapped in this study. The *longest arrow* (*asterisk*) represents the *orf138-orfB* cotranscript present in sterile plants. The *shorter arrow* (*dot*) represents the major *orfB* RNA of plants with the Ogura-related cytoplasm. *Arrowheads* show the position of primers used in cRT-PCR, the internal primer was used for cDNA priming (cDNA priming), the external primers for amplification (cRT-F & cRT-R). **c** Precise mapping of the ends of the new *orfB* RNA (*dot* in Figs. 3, 4b). The results of the sequencing of cRT-PCR products are summarized: the *vertical bars* above the sequence indicate the positions of the 5′ ends of RNA. The lengths of the bars are proportional to the number of clones with the considered 5′ end, given above the bars. The *Xba*I site corresponding to the end of probe D (see Fig. 1) used in RNA hybridization analysis (Fig. 3) is shown in *bold*

sizes were obtained, as estimated by amplification, and the clones could be grouped into three size classes. Class I corresponded to the major cRT-PCR product in plants with the Ogura cytoplasm. Class II corresponded to the major cRT-PCR product in plants with the wild Ogura-related cytoplasm. Class III clones had inserts shorter than those of classes I and II. We obtained the sequences of five clones for class I, 15 clones for class II, and 30 clones for class III. In all three classes, the 3′ end of the RNA corresponded to the 3′ end of the previously mapped *orf138-orfB* cotranscript (Bonhomme et al. 1992; Bellaoui et al. 1997). For classes I and II, the 5′ ends of the RNAs determined from the different clones were mapped within a few bases of the DNA sequence. Class I corresponded to full-length *orf138-orfB* cotranscripts (asterisk), as previously mapped (Bonhomme et al. 1992; Bellaoui et al. 1997). Class II corresponded to RNAs with a 5′ end in the middle of the *orf138* coding sequence (Fig. 4b), consistent with the length of the major *orfB* RNA in plants with the Ogura-related cytoplasm (dot). For the third class, corresponding to the smallest inserts, the 5′ ends of the RNAs were scattered in the second half of the *orf138* coding sequence, with no obvious major position.

Western blots of protein extracts from plants carrying the wild Ogura-related cytoplasm with an antibody raised against the ORF138 protein (Grelon et al. 1994) confirmed that these plants produced no detectable ORF138 protein (data not shown).

Discussion

An Ogura-related cytoplasm of ancient origin is present in European populations of *R. raphanistrum*

According to theoretical models, the presence of a sterility-inducing cytoplasm leads to the selection of restorers of fertility. The discovery of restorers for the Ogura CMS in natural French populations of *Raphanus raphanistrum* (Eber et al. 1994), naturally led to searches for the Ogura CMS in these populations. However, we found no female plants in natural populations of *R. raphanistrum*, including the population in which these restorers were first detected. Fortunately, the mitochondrial gene responsible for the Ogura CMS is known and can be readily detected by PCR. We searched for the *orf138* gene in wild plants or their maternal descent and identified a few plants carrying this gene. Four of the 17 populations studied included individuals with the *orf138* gene. As the populations sampled were not evenly distributed geographically, it is difficult to determine the distribution of the Ogura-related cytoplasm. However, it is clear that, when present, this cytoplasm is not frequent. In contrast, Murayama et al. (2004) reported a high frequency of the *orf138* gene in populations of wild radish in Japan, most of which were gynodioecious. The situation in European populations of *R. raphanistrum* therefore clearly differs from that of Japanese populations of wild radish, at least as far as the Ogura CMS is concerned. The low frequency of *orf138* in our populations suggests that this cytoplasm has either only recently been introduced or is an ancient cytoplasm destined to become extinct. *Brassica* hybrids with the Ogu-INRA cytoplasm, derived from the Ogura cytoplasm, have been cultivated in France for a decade. This might suggest acquisition of the *Brassica* cytoplasm of hybrids by wild *Raphanus raphanistrum* through spontaneous interspecific crosses. However, this possibility can be ruled out because the Ogu-INRA cytoplasm has characteristics absent from the wild Ogura-related cytoplasm. Firstly, plants with the Ogura cytoplasm have a second copy of the *tRNAfMet* gene; plants with the Ogu-INRA cytoplasm do not (Bonhomme et al. 1999). Secondly, the Ogu-INRA cytoplasm includes a plastid genome from *Brassica* species (Pelletier et al. 1983; Bonhomme et al. 1999), whereas the plastid sequences found in the Ogura-related cytoplasm were identical to those of the original radish Ogura cytoplasm. Thirdly, the intergenic sequence between *orf138* and *orfB* in the radish Ogura and Ogura-related cytoplasms differs slightly from that of the Ogu-INRA cytoplasm. In addition, the *orf138* coding sequence of wild *R. raphanistrum* plants differs at position 99 (silent polymorphism) from the Ogura sequence present in *Brassica* hybrids. Interestingly, the sequence of the *orf138* gene present in the novel Ogura-related cytoplasm described here is identical to the type B sequence in wild and cultivated radishes (Yamagishi and Terachi 2001). This sequence was most frequent between the wild radish and *R. raphanistrum* plants studied by these authors, suggesting that type B is the ancestral sequence for *orf138*. These data, and the presence of fertility restorers in European populations of *R. raphanistrum*, are consistent with the Ogura-related cytoplasm in these populations being ancient rather than recently introduced.

The European Ogura-related cytoplasm is very similar to the original Ogura cytoplasm, but does not cause sterility

Terachi et al. (2001) described three types of cytoplasm in wild and cultivated radishes that differed in terms of

their *orfB* 5′ flanking sequences. One of these sequences was found to be strictly linked to *orf138* and found in Ogura and Ogura-related cytoplasms. These authors suggested that the *orf138* and *orfB* genes became associated only once in the history of radish cytoplasms. However, this assertion was based on observation of the *orf138-orfB* locus only, and the sequencing results obtained were not completely consistent with those of other studies (see the discussion in Terachi et al. 2001). Our analysis of the *orf138-orfB* locus in European Ogura-related cytoplasm yielded a sequence identical to that reported by Terachi et al. (2001). We investigated plastid genome polymorphism in the prospected European populations, and clearly identified a sequence specific for the Ogura-related cytoplasm for both plastid regions studied (ESM). This sequence was identical to that of the original Ogura cytoplasm, taken as a reference, indicating that the *orf138-orfB* locus probably does have a monophyletic origin. In addition, our DNA hybridization analysis of this locus showed no difference between the original Ogura cytoplasm and the European Ogura-related cytoplasm (Fig. 2).

We showed, by crosses with tester genotypes, that fertility restorers were not fixed in the prospected natural populations, as some individuals were not homozygous for restorers. However, the small numbers of seeds obtained from these crosses precluded a genetic analysis of restoration. In addition, we did not include in this study crosses involving plants with cytoplasms different from the Ogura-related cytoplasm. Further genetic studies are required to determine the number of restoration loci present in French natural populations of *R. raphanistrum*, the frequency of restorer alleles, and their possible linkage to the identified *Rfo* locus (Brown et al. 2003; Desloire et al. 2003; Koizuka et al. 2003). Nevertheless, two backcrosses with the maintainer radish tester line failed to reveal male sterility in the maternal progenies of plants heterozygous for fertility restorers. This unexpected result shows that the newly identified Ogura-related cytoplasm does not induce sterility. As the type B *orf138* coding sequence is associated with male sterility-inducing cytoplasms in Japanese wild radishes (Yamagishi and Terachi 1997), we concluded that this silent polymorphism is not the cause of the lack of sterility phenotype. We therefore analyzed *orf138* expression in male fertile plants with the European Ogura-related cytoplasm.

The *orf138* transcript is interrupted by a processing event favored in the wild Ogura-related cytoplasm

RNA hybridization analyses clearly showed that the expression profile of the *orf138-orfB* locus differed between the two cytoplasms (Fig. 3). Whereas the *orf138-orfB* cotranscript (Bonhomme et al. 1992; Bellaoui et al. 1997) accumulates in Ogura plants, a smaller RNA was the major *orfB* RNA detected in plants with the Ogura-related cytoplasm. This RNA was not detected with a probe spanning the 5′ part of the *orf138* sequence, indicating that this RNA does not contain the entire *orf138* coding sequence. Mapping of the 3′ and 5′ ends of this RNA indicated that its 3′ end was the same as that of the *orf138-orfB* mRNA in Ogura plants, but that its 5′ end lay within the *orf138* coding sequence. We have shown that the *orf138-orfB* locus is transcribed from a promoter upstream from the *fMtRNA* gene, and that the 5′ end of the *orf138-orfB* mRNA undergoes processing (Bellaoui et al. 1997). It therefore appears likely that a different, or additional, processing event produces the 5′ end of the major *orfB* mRNA in the Ogura-related cytoplasm. A small amount of *orf138-orfB* Ogura mRNA was detected in plants with the Ogura-related cytoplasm, and, reciprocally, a small amount of the truncated *orfB* mRNA was detected in Ogura plants (Fig. 3). This observation suggests that the observed differences in the expression profile of this locus are based on a quantitative, rather than qualitative mechanism. The interruption of a sterility gene transcript by a maturation event is very similar to the action of a fertility restorer (Hanson and Bentolila 2004). However, in this case, the processing event depends on cytoplasm type rather than on a nuclear locus. Firstly, the RNA mapped in this study was the major *orfB* mRNA only in plants carrying the European Ogura-related cytoplasm, and never in plants carrying the Ogura cytoplasm, although the introduction of a restorer from a wild plant rendered these plants fertile (data not shown). Secondly, this *orfB* mRNA also predominated in plants from the second backcross with the L7F maintainer line (M2, M4, and M5 in Fig. 3). As these three plants derived from second backcrosses with the L7F maintainer line and presented the same *orfB* transcription profile, this profile is very unlikely to result from the residual presence of a restorer allele in all three plants. A similar situation has been described in *Arabidopsis thaliana*, in which *cox3* transcript maturation differs in the C24 and Col0 cytoplasms (Forner et al. 2005). However, in this case, differences in distant upstream sequences provided a possible explanation for the differences in maturation sites. In the case presented here, we have identified no sequence difference likely to account for our observations. It is unlikely that the single nucleotide substitution inside the *orf138* gene affects the processing of the *orf138* RNA because this substitution is also present in male-sterility inducing cytoplasms of

Asian genotypes (Yamagishi and Terachi 1997). Nevertheless, no sequencing was carried out outside the expressed region and, although DNA hybridization analysis detected no differences between the Ogura and Ogura-related cytoplasms, these two cytoplasms may differ in other regions of the mitochondrial genome.

Small amounts of *orf138-orfB* mRNA were detectable in plants with the Ogura-related cytoplasm, but no ORF138 protein could be detected in protein extracts from these plants (data not shown). This small amount of mRNA therefore appears to be insufficient for protein production, accounting for the lack of sterility induction by the wild cytoplasm.

We conclude that the Ogura-related cytoplasm of wild plants in European natural populations carries an *orf138* locus of the "ancestral" type that has lost its ability to induce male sterility due to processing of its transcript, disrupting the *orf138* coding sequence. The presence of fertility restorers in these populations strongly suggests that this cytoplasm was once able to induce male sterility. To our knowledge, such a situation is unprecedented in the CMS systems generally studied in natural populations, because the sterility genes have not been identified and cannot be followed independently of sterility phenotype. Theoretical predictions concerning CMS maintenance in gynodioecious populations suggest that a loss of sterility induction would be expected when restorer alleles become fixed in the population (Bailey et al. 2003). In the populations studied here, it is clear that the restorers are not fixed. However, we cannot rule out the possibility that they were once fixed rendering the sterility-associated cytoplasm neutral and allowing unknown cytoplasmic changes to impair *orf138* expression, resulting in the observed male fertility "reversion". The low frequency of the wild Ogura-related cytoplasm in European populations suggests that there may have been a subsequent drift effect on this cytoplasm. Murayama et al. (2004) found that restorers were not fixed in Japanese wild radish populations, and suggested that this may be due to a cost of restoration—a decrease in the fitness of plants carrying the fertility restorer but not the sterility-inducing cytoplasm. We do not yet know whether the restorers present in European populations are the same as those present in Japanese populations, and cannot evaluate the possible costs associated with these restorers. However, assuming that European restorers do have a cost, they would be expected to accumulate mutations subject to possible selection after the "reversion" of the Ogura-related cytoplasm. It should be possible to test this hypothesis by comparing restorer and non-restorer alleles from European populations and analyzing their phylogenic relationship. The original situation described here provides an opportunity for experimental studies of the fate of restorer genes in the absence of the corresponding male sterility cytoplasm. The striking difference between the situations of the Ogura CMS system in Japanese and European natural populations is difficult to understand at this stage. It would be interesting to compare the demographic situations of the populations, to establish the evolutionary scenario underlying this "chronicle of a death foretold".

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