Theor Appl Genet (2000) 100:949–955

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Genetic analysis of fertility restoration and accumulation of ORF125 mitochondrial protein in the kosena radish (*Raphanus sativus* cv. Kosena) and a *Brassica napus* restorer line

Received: 13 August 1999 / Accepted: 16 September 1999

Abstract The genetics of fertility restoration (Rf) of kosena radish CMS has been characterized. The kosena CMS-Rf system is genetically the same as that of the ogura CMS-Rf system. Two dominant genes that act complementary to the restoration of fertility control fertility restoration in kosena CMS. One allele (*Rf1*) is associated with accumulation of the CMS-associated protein, ORF125. The interaction of *Rf1* and another allele (*Rf2*) was essential for the restoration of fertility in radish, whereas *Rf1* alone was sufficient for the complete restoration of fertility in the *B. napus* kosena CMS cybrid.

Key words. Fertility Restoration (Rf) · Cytoplasmic male sterility (CMS) · *Raphanus sativus* L. · *Brassica napus* · Mitochondria

Introduction

Cytoplasmic male sterility (CMS) and its fertility restoration (Rf) system have been studied intensively in many crops, such as maize, sunflower, rice, *Brassica napus*, etc. (Schnable and Wise 1998). In addition to facilitating the commercial exploitation of CMS-Rf systems, detailed studies of CMS and Rf genes provide us with information that increaes our understanding nuclear-cytoplasmic interactions (Budar 1998).

Communicated by D.R. Pring

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In *B. napus*, several mitochondrial open reading frames (orfs) have been identified as CMS-associated genes. The *orf138* gene, which has been isolated as an ogura CMS (ogu CMS)-associated gene, is one of the most intensively studied "CMS genes" (Makaroff 1995). The ogu CMS-associated gene has been cloned by comparing the mitochondrial (mt) DNA of the *B. napus* ogu CMS cybrid with its fertile revertant (Bonhomme et al. 1992). The gene encodes a 19 kDa protein that accumulates in the mitochondrial membrane (Bonhomme et al. 1992; Grelon et al. 1994).

Previously, we transferred a radish CMS-Rf system to *B. napus* by donor-recipient protoplast fusion (Sakai and Imamura 1992; Sakai et al. 1996). We termed the CMS as kosena CMS (kos CMS). An analysis of the kos CMS mtDNA revealed that kos CMS carries *orf125* (Iwabuchi et al. 1999), which encodes a 17-kDa protein and has a sequence homologous to that of *orf138*, except for two amino acid substitutions and a 39-bp deletion in the *orf138* coding region. The accumulation of ORF125 and ORF138 is associated with the CMS phenotype in *B. napus* (Grelon et al. 1994; Iwabuchi et al. 1999), and the *Rf* gene regulates protein expression at the translational level (Koizuka et al. 1998).

In comparison with molecular-based studies of the CMS-Rf system, genetic analysis of this system has not been well studied. Our previous report suggested that ogu and kos CMS can be restored by pollen from the same restorer plant, although no detailed genetic analysis was performed. It has been reported that several *Rf* alleles may be associated with the fertility restoration in ogu CMS (Bonnet 1975; Nieuwhof 1990), however, the exact number of *Rf* alleles has not been clearly determined.

In this paper, we describe a genetic analysis of nuclear restoration in radish kos CMS and *B. napus* kos cybrids. Through this analysis we were able to reveal that two dominant alleles of *Rf* genes that act complementary are necessary for complete fertility restoration in radish. Moreover, a comparison of the restoration processes between kos and ogu CMS suggested that these CMS sys-

Table 1 Characteristics of radish and *Brassica napus* plants used in this study

	Cytoplasms	Phenotypes	Origins
Radish			
KA2	kos cms	Sterile	Kosena CMS line (Kos A)[a]
KA11	kos cms	Sterile	Kosena CMS line (Kos A)[a]
KA14	kos cms	Sterile	Kosena CMS line (Kos A)[a]
(KA2×KC1)-1	kos cms	Sterile	A sterile F_1 plant between KA2 and KC1
KC1	Normal cytoplasm	Fertile	Kosena Rf line (Kos C)[b]
KC6	Normal cytoplasm	Fertile	Kosena Rf line (Kos C)[b]
KC6-4	Normal cytoplasm	Fertile	A plant derived from KC6 self-pollination
Yuan10	Normal cytoplasm	Fertile	cv. Yuan hong (a Chinese variety)
Yuan10-3	Normal cytoplasm	Fertile	A plant derived from Yuan 10 self-pollination
Ogu-CMS	ogu cms	Sterile	A CMS plant derived from Ogura CMS line
Ogu-2	ogu cms	Fertile	An Rf plant of Ogura CMS line
B. napus			
B. napus restorer line (*RfRf*) referred as BRf	A CMS cybrid (same as 18-3)	Fertile	Selected from BC_5S_5 generation of a fertile fusion product between CMS cybrid 18-3 and Kos C (Sakai et al. 1996)
18-3	A CMS cybrid	Sterile	Protoplast fusion between Kos A and *B. napus* (Sakai and Imamura 1992)
12-9	A CMS cybrid	Sterile	Protoplast fusion between Kos A and *B. napus* (Sakai and Imamura 1992)
Alloplasmic ogu	ogu cms	Sterile	*B. napus* nuclear substitution line of ogura CMS cytoplasm[c]

[a] Kos A line was isolated from cv. Kosena and maintained by the Kos B line. Kos B originated from a single plant that was isolated from a Kosena population as a maintainer

[b] Kos C line was also isolated from the same population of cv. Kosena as a restorer line

[c] Seeds were kindly provided by Professor Steffanson, University of Manitoba, in 1987

tems are closely related functionally in addition to being similar in their nucleotide sequences.

Materials and methods

Plant materials

Kosena CMS (kos CMS) and Kosena Rf (kos Rf) lines isolated by Ikegaya (Ikegeya 1986) were used. Table 1 summarizes the origin and features of the materials analyzed in our experiments. Detection of the cytoplasms was performed by polymerase chain reaction (PCR) that determined the presence or absence of CMS-associated gene *orf125*/*orf138* sequences. Kos CMS plants were randomly selected from the kos CMS line and were maintained by crossing them with the kos maintainer (Kos B). Rf plants were randomly selected from kosena Rf line (Kos C) and a population of Chinese cv. Yuan hong (Yuan 10). Seeds of Ogura radish were kindly provided from the horticulture laboratory of Iwate University. All crosses were made with plants grown in a growth chamber under a 16-h (day) photoperiod and day/night temperature of 23°/18°C. Each CMS plant was hand-pollinated with pollen from individual Rf plants. Eight fertile siblings derived from the cross between (KA2/KC1)-1 and Yuan 10-3 were self-pollinated in bags and treated with CO_2 gas to break the self-incompatibility of the plants. The self-pollinated seeds were grown in a field in the autumn of 1996, and the fertility of the flowers was checked in the spring of 1997. Male-sterile flowers with completely aborted anthers shedding no pollen can be easily distinguished from fertile flowers in both the greenhouse and under field conditions.

Brassica napus kos CMS cybrid and kos-Rf lines were derived from protoplast fusion experiments (Sakai and Imamura 1992; Sakai et al. 1996). Two kos CMS cybrids (12-9 and 18-3) were regenerated from independent calli and had 38 chromosomes in the R_0 generation. Analysis of mtDNA digestion patterns indicated that the two kos CMS cybrids have different mtDNA configurations (Sakai and Imamura 1993). These kos CMS cybrids were maintained by three to six times backcrosses with *B. napus* cv. Westar pollen grains. Male-sterile flowers of the two cybrids have completely aborted anthers shedding no pollen grains. To date, the male sterility is stable in both spring- and winter-type *B. napus* nuclear backgrounds (data not shown). Alloplasmic ogura CMS carrying the *B. napus* nucleus and radish cytoplasm were produced by nine times backcrosses with *B. napus* cvs. Marnoo and Westar. The seeds were kindly provided by Professor Steffanson, University of Manitoba (Winnipeg, Canada).

Protein extraction and Western blotting analysis

Inflorescences of radish and *B. napus* plants were ground in 50 m*M* Tris-HCl (pH 7.5), 2% (w/v) SDS. The homogenates were centrifuged at 15 000 *g* for 10 min at 4°C, and the resulting supernatants were used for Western blotting analysis. A Bio-Rad protein assay kit (Bio-Rad) was used to estimate the total protein concentration using IgG as a standard. Aliquots of 10–15 µg of total protein were separated on 12% SDS-PAGE and then blotted onto an Immobilon-P membrane (Millipore) according the method described by Sambrook et al. (1989). The blotted membrane was cut into two halves – one for higher molecular-weight (MW) proteins was used for detecting the mitochondrial ATPase α-subunit (ATPA); the other for lower MW proteins was used for detecting the ORF125 protein. Immunodetection for ORF125 protein and ATPA were done as described by Iwabuchi et al. (1999) except for an ECL Western blotting analysis system (Amersham) used for the detection of ATPA. The monoclonal antibody against ATPA was kindly provided by Dr. Thomas Elthon (Luethy et al. 1993).

RNA isolation and Northern blot analysis

Inflorescences of fertility-restored (Rf) and CMS plants were ground in liquid nitrogen in a mortar. The powder was suspended in 4 *M* guanidinium thiocyanate, 25 m*M* sodium citrate, pH 7, 0.5% (w/v) sodium lauryl sarkosyl, and 1% (v/v) 2-mercaptoethanol and then centrifuged at 15 000 *g* for 10 min. The resulting supernatant was extracted twice with chloroform/isoamylalchol (24:1). The aqueous layer was collected, and a 1/10 volume of 3 *M* sodium acetate (pH 5.2) and an equal volume of isopropanol were added. After centrifugation at 15 000 *g* for 20 min the pellet was dissolved in sterile distilled water. Higher-molecular-weight RNA was precipitated by the addition of a lithium chloride solution to a final concentration of 2 *M*. Total RNA was dissolved in a small volume of sterile distilled water and stored at –80°C until the Northern blot analysis. A 10-µg portion of total RNA was denatured, electrophoresed on an agarose/formaldehyde gel, and transferred onto a Hybond-N membrane (Amersham) using 20× SSC as a transfer buffer. Hybridization was carried out with [^{32}P]-dCTP-labeled *orf125* coding region as a probe according to the manufacturer's instruction. The filters were washed once in 2× SSC, 0.1% SDS for 30 min at 42°C, followed by two 30-min washes in 0.1× SSC, 0.1% SDS at 60°C. The signal was visualized by autoradiography.

Results

Fertility restoration manners in kos CMS radish

In a previous report, we indicated that the fertility of ogu and kos CMS was restored by crossing with the same restorer plants, and these CMS cytoplasms could be maintained by crossing with the same maintainer line (Iwabuchi et al. 1999). We also confirmed that the fertility of kos CMS was also restored by the ogura Rf plant, Ogu-2 (Table 2). These results indicated that the restoration and maintenance of kos CMS were the same as that of ogu CMS radish. To understand fertility restoration systems in kos radish (refer to Table 1 for the names of each plant used in the studies), we crossed several Rf plants to kos CMS plants. All F_1 progeny from crosses of KA11 and KC6 and crosses of KA14 and Yuan10 were fertile, while F_1 plants between KA2 and KC1 segregated for male-sterile and male-fertile plants (Table 2). These results indicated that KC1 was heterozygous and that both KC6 and Yuan10 were homozygous for the *Rf* allele. To confirm these results, we crossed a segregated CMS plant, (KA2/KC1)-1, with pollen from the three individuals of Rf progeny, KC6-4, Yuan10-3, and Ogu-2. The crosses of (KA2/KC1)-1 and KC6-4 resulted in no restoration of male fertility, whereas all of the progeny from crossing (KA2/KC1)-1 with Yuan 10-3 or Ogu-2 were fertile. These results are inconsistent with results obtained in the F_1 generation that KC6 should be homozygous for the *Rf* allele.

To analyze the genotype of the *Rf* allele in kos CMS, we developed eight families from the self-pollination of the fertile plants derived from the test-cross of (KA2/KC1)-1 and Yuan10-3 (Table 3). The eight families were expected to segregate 3:1 (male fertile: sterile) if the fertility restoration of the kos CMS was controlled by a single dominant allele. All the populations (KE1–KE8) exhibited the expected 3:1 segregation ratio except for KE1 and KE4. The segregation ratios of KE1 and KE4 exhibited statistically significant deviations from the expected 3:1 ratio and instead yielded a 9:7 ratio. That is the expected ratio for fertility restoration regulated by two independent dominant alleles. From these results, we hypothesize that two dominant *Rf* alleles might be required for restoration of the fertility of kos CMS cytoplasm in radish. The genotypes of the eight plants were classified into two groups by the segregation ratios: (1) homozygous for one of the *Rf* alleles (segregated 3:1 in the eight populations including KE2, KE3, and KE5–KE8) and (2) heterozygous for both *Rf* genes (segregated 9:7 in the populations including KE1 and KE 4).

Table 2 Segregation of male fertility and sterility in F_1 and test-crosses in kos and ogu CMS with putative radish Rf lines

Generations	Crosses		Male fertility	
	CMS	Rf	Fertile	Sterile
F_1	KA2	KC1	6	3
	KA11	KC6	22	0
	KA14	Yuan 10	25	0
Test-cross	(KA2/KC1)-1	KC6-4	0	8
	(KA2/KC1)-1	Yuan10-3	44	0
	(KA2/KC1)-1	Ogu2[a]	16	0
F_1	ogu CMS	Yuan10-3	9	0

[a] Fertility restoration of the cross between ogu CMS and Ogu 2 was examined previously (data not shown)

Table 3 Genetic analysis of selfed populations derived from eight individual plants of the test-cross between a CMS ((KC2/KA1)-1) and an Rf plant (Yuan 10-3)

Populations	Total no. of plants	Number of plants with the indicated male-fertility status		Proposed ratio	X^2 value	*P* value
		F	S			
KE1	254	138	116	9:7	0.38	0.54
KE2	169	136	33	3:1	2.70	0.10
KE3	91	73	18	3:1	1.32	0.25
KE4	315	192	123	9:7	2.83	0.09
KE5	169	133	36	3:1	1.23	0.27
KE6	461	343	118	3:1	0.09	0.77
KE7	373	285	88	3:1	0.39	0.53
KE8	261	192	69	3:1	0.29	0.59

Regulation of *orf125* expression by *Rf* alleles

As described in the Introduction we have identified a kos CMS-associated gene, *orf125*, that is a homolog of the ogu CMS-associated gene, *orf138*, and considerable reduction in ORF125 protein accumulation was observed in the fertility-restored *B. napus* cybrid (Iwabuchi et al. 1999). Preliminary results suggested that only one dominant gene is required for the fertility restoration in the *B. napus* CMS cybrid (Sakai et al. 1996). Based on our present results, we assume that two independent alleles are required for fertility restoration, with one of the two alleles regulating the level of ORF125 protein accumulation in radish. In order to verify this assumption, ORF125 protein levels in all of the plants of KE1 and KE3 populations were examined by Western blotting. Typical results of Western blotting patterns are shown in Fig. 1. The absence of accumulation of the ORF125 (17 kDa) protein was consistent with fertility restoration in the KE3 population, which showed a 3:1 (male fertile: sterile) segregation ratio (Fig. 1A). On the other hand, the presence or absence of this protein accumulation was not consistent with fertility restoration in the KE1 population, which showed a 9:7 (male fertile: sterile) segregation ratio (see asterisks Fig. 1B). Accumulation of ATPA was almost the same between these plants, as determined from Western blotting using monoclonal antibody against this protein (data not shown). Table 4 summarizes the results of the segregation ratio concerning the accumulation level of ORF125 protein in the two populations. The 3:1 (plants with ORF125: plant without ORF125) segregation ratio was even observed in the KE3 populations which showed a 9:7 segregation ratio for male fertility. This is consistent with the expected ratio if one *Rf* allele dominantly regulates the level of ORF125 accumulation. Therefore, we refer to this allele as *Rf1*. Genetic analysis of the radish population also

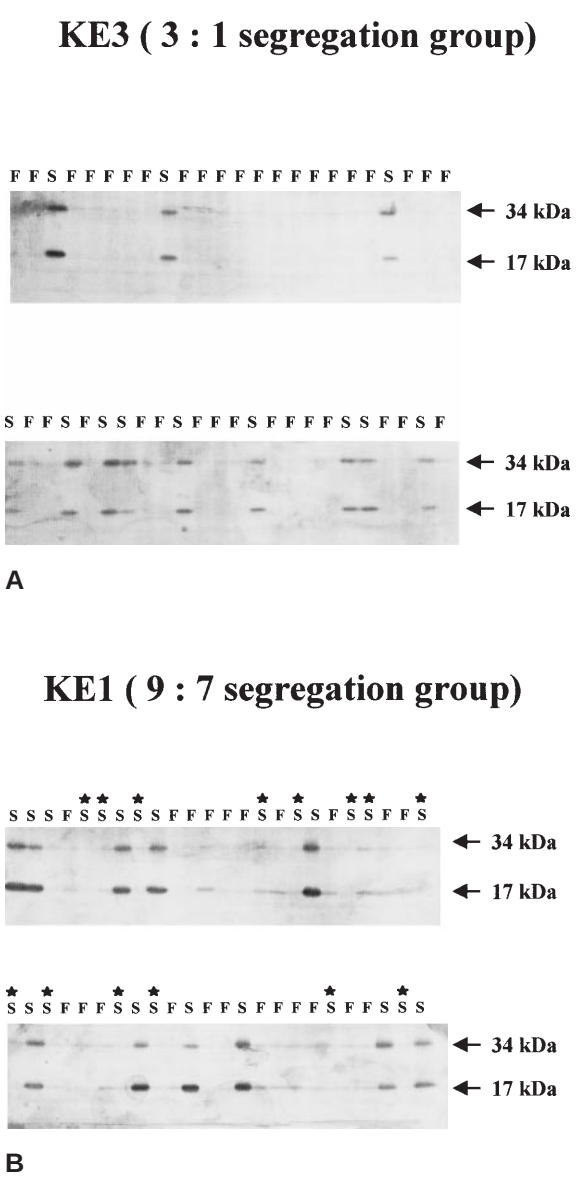

Fig. 1A, B Western blot analysis of ORF125 protein in two families of radish (see Table 3). **A** Analysis of the KE3 family that showed a 3:1 segregation ratio for male-fertile (*F*) and male-sterile (*S*) flowers. **B** Analysis of the KE1 family that showed a 9:7 segregation ratio. *Asterisks* indicate male-sterile plants in which the accumulation of ORF125 protein was restricted

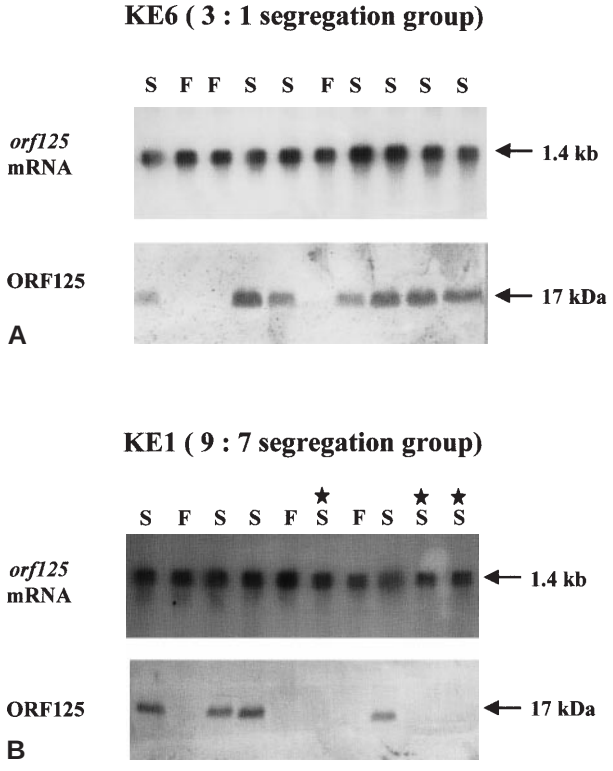

Fig. 2A, B Northern blot (*upper panels*) of *orf125* and Western blot analysis (*lower panels*) of ORF125 protein in different segregation groups of the KE6 and KE1 populations (see Table 3). **A** Analysis of randomly selected plants from the KE6 family that revealed a 3:1 segregation ratio for male-fertile (*F*) and male-sterile (*S*) flowers. **B** Analysis of randomly selected plants from the KE1 family that revealed a 9:7 segregation ratio for male-fertile (*F*) and male-sterile (*S*) flowers. *Asterisks* indicate male-sterile plants in which the accumulation of ORF125 protein was restricted

Table 4 Segregation ratio for presence or absence of the ORF125 protein in the selfed populations of the test-cross

Populations	Segregation ratio for fertility restoration	Number of plants with (+) or without (–) ORF125 accumulation				
		–	+	Total	X^2 value for 3:1	*P* value
KE1	9:7	189	62	251	0.01	0.91
KE3	3:1	73	18	91	1.32	0.25

Table 5 Segregation for fertility restoration and ORF125 accumulation in the cross between *B. napus* CMS cytoplasms and homozygous Rf line

Cytoplasms	Nuclear genotypes of male parents	Crosses	Fertility (no. of plants with *orf125* decrease)		
			F	S	PF[c]
Kos-cybrid 18-3	*Rf1*/*Rf1*	CMS×BRf[a]	25 (25)[b]	0	0
Kos-cybrid 18-3	*Rf1*/*rf1*	(CMS×BRf[a]) self	24 (24)	15 (0)	0
Kos-cybrid 12-9	*Rf1*/*Rf1*	CMS×BRf[a]	3 (3)	3 (3)	7 (7)
Alloplasmic ogu	*Rf1*/*Rf1*	CMS×BRf[a]	0	31 (31)	1 (1)

[a] Origin of the BRf plant is described in Table 1
[b] Numbers in parentheses are the number of plants showing a reduced level of ORF125 protein by Western blotting (see Fig. 3)
[c] PF indicates plants with partially fertile flowers. Several types of flowers were observed in all of the RF plants and these were described as follows: (1) flowers that have completely male-fertile anthers; (2) flowers that have completely sterile anthers; (3) flowers that have both fertile and sterile anthers in the same flower organ. These three types of flowers were present in the same PF plants

suggested that not only *Rf1* but also another dominant *Rf* allele is required for the restoration of fertility. We provisionally refer to this *Rf* allele as *Rf2*. The presumed genotypes of the eight plants shown in Table 2 that segregated in a 3:1 (male fertile: sterile) ratio were *Rf1*/*rf1*, *Rf2*/*Rf2* and those that segregated in a 9:7 (male fertile: sterile) ratio were *Rf1*/*rf1*, *Rf2*/*rf2*.

An antibody against ORF125 protein also recognized 34- and 51-kDa proteins (data not shown) when ORF125 protein (17 kDa) was identified in the radish inflorescence. This phenomenon was also observed in the *B. napus* kosena CMS cybird (Iwabuchi et al. 1999). In ogura CMS radish, ORF138 protein and additional 36- and 54 kDa proteins were detected using an antibody against the ORF138 protein (Krishnasamy and Makaroff 1994). The nature of these additional bands has yet to be investigated.

To examine the effects of the *Rf2* allele on the expression of *orf125*, we performed RNA blot analysis using total RNA isolated from inflorescences of randomly selected fertile and sterile plants among KE6 and KE1 populations (see upper panel of Fig. 2A and B). Western blot analysis was also done using total protein extracts from the same inflorescences (lower panel of Fig. 2A

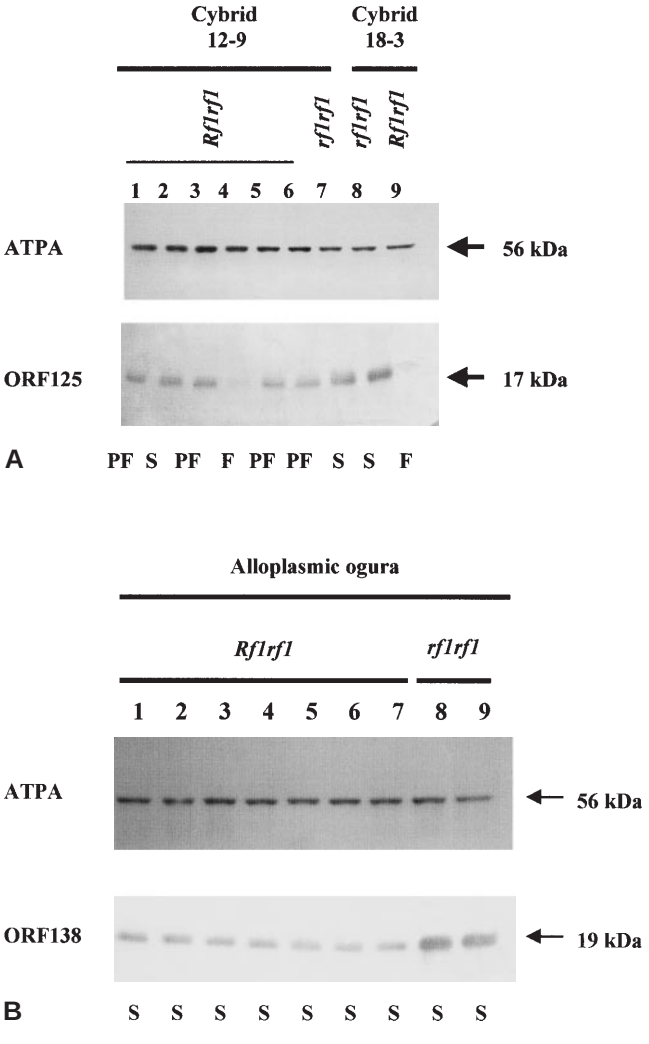

Fig. 3A, B Western blot analysis of expression in the kos **A** and ogu **B** CMS *B. napus* that are heterozygous (*Rf1*/*rf1*) and recessive homozygous for (*rf1*/*rf1*) fertility restorer genes. Cytoplasm types are described in the *upper part* of the lanes (for details, see Table 1) and genotypes of each of the analyzed plants are shown in each *column*. Mitochondrial ATPA was used as an internal control in the experiment. The flower morphology of each plant tested is shown at the *bottom* of the photograph: *S* Male-sterile plants, *PF* Partial fertile plants, *F* Fertile plants. For a detailled description of the phenotype of the paritally fertile plants see in Table 5

and B). The presumed genotypes of the fertile plants from the KE6 population are homozygous for the *Rf2* allele (*Rf1*-/*Rf2Rf2*) and that from the KE1 population is *Rf1*-/*Rf2*-. The 1.4-kb transcript was present in all the plants, indicating that the *Rf2* allele had no visible effects on the expression of *orf125* gene at the transcriptional level.

Genetic analysis of radish *Rf* allele in *B. napus*

As described above, a single dominant *Rf* allele was introduced by cell fusion into *B. napus*. We examined the fertility restoration manner of three different CMS cytoplasms (18-3, 12-9 and alloplasmic ogu) in *B. napus* by crossing these with *B. napus* homozygous for the *Rf* allele (BRf, see Table 1). When CMS cybrid 18-3 was crossed with a homozygous BRf plant, all the F_1 progeny became male-fertile. Fertility restoration of the flowers was complete in all of the individual flowers observed (data not shown). The accumulation or decrease in ORF125 protein level in the inflorescences was completely consistent with fertility in both the F_1 plants and F_2 plants of cybrid 18-3 and BRf (Fig. 3A, lanes 8 and 9, Table 5). On the other hand, the BRf plant could not completely restore the F_1 progeny of CMS cybrid 12-9 (Table 5). Some of these F_1 progeny have partial fertile phenotypes (Table 5). In spite of incomplete fertility restoration, the accumulation of the ORF125 protein in these F_1 progeny was considerably restricted compared to that in the F_1 progeny from normal *B. napus* (*rf1*/*rf1*) and CMS cybrid 12-9 (lanes 1–7 in Fig. 3A). Similar results were obtained in the crosses of the BRf and alloplasmic ogu CMS plant (Table 5). The ogu CMS-associated ORF138 protein was also slightly reduced in the F_1 plants (*Rf1*/*rf1*) compared to the CMS plant (*rf1*/*rf1*) (Fig. 3B). So far we have observed that the *Rf1* gene is a prerequisite but singly not sufficient for fertility restoration in the 12-9 and alloplasmic ogu cytoplasms.

Discussion

The data presented here show that restoration of pollen fertility in kos cytoplasm radish is controlled by two unlinked dominant alleles, *Rf1* and *Rf2*. It has been reported that at least several dominant or recessive nuclear factors are involved in fertility restoration of ogu CMS (Bonnet 1975, Neiuwhof 1990). Neiuwhof (1990) reported that several minor genes are involved in the control of fertility in ogu CMS, but there is still no clear evidence for this. However, CMS genotypes can be distinguished as *Rf1*-/*rf2rf2* or *rf1rf1*/– by analyzing the levels of ORF125 protein accumulation; consequently, the exact number of alleles required for restoration can be concluded.

Reduction in the level of the ORF125 protein, which is a product of the kos CMS-associated gene, *orf125*, is tightly correlated with the *Rf1* genotype in radish (Table 4 and Fig. 1). These phenomena were also observed in *B. napus* cybrid plants (Table 5 and Fig. 3A). Therefore, we assume that the *Rf1* allele dominantly regulates the level of ORF125 accumulation. Expression of the *orf125* transcript in radish flower buds was almost the same between sterile and fertility-restored plants, thus the *Rf1* allele might be regulated at the post-translational level (Koizuka et al. 1998). More extensive studies are required to determine just how the *Rf1* allele regulates *orf125* expression in radish. Accumulation of ogura CMS-associated ORF138 protein was also reduced in the F_1 plants from a cross of *B. napus* homozygous for *Rf1* gene and alloplasmic ogu CMS, although fertility restoration was incomplete (Fig. 3B, Table 5). These results suggest that *Rf1* and the fertility restoration gene for ogura radish (*Rfo*: Delourme et al. 1994) might be functionally identical.

In contrast, the *Rf2* genotypes have no visible effects at the transcriptional and translational levels of *orf125* (upper panel in Fig. 2B). In maize T-cytoplasm the restoration of fertility from CMS requires the combined action of the dominant alleles of two nuclear genes, *rf1* and *rf2* (Pring and Lonsdale 1989). Though the accumulation of the CMS-associated protein URF 13 is reduced in plants carrying *Rf1*, there is no molecular phenotype associated with the *rf2* allele. The cloned maize *rf2* gene encodes a homolog of mammalian mitochondrial aldehyde dehydrogenases (Cui et al. 1996). *Rf2* is thought to act indirectly with URF13. The complementary actions of *Rf1* and *Rf2* diminish the deleterious effect of the T-cytoplasm in male flower organs (Cui et al. 1996). In kos CMS cytoplasm, though the *Rf2* gene in radish has not yet been identified, it might have similar functions to those of the maize *Rf2* gene.

The phenotype of the fertility restoration of *B. napus* cybrid 18-3 was consistent with the decrease in the accumulation levels of the ORF125 protein. No exception has as yet been observed. We therefore conclude that restoration of pollen fertility in CMS cybrid 18-3 is controlled only by the *Rf1* gene. The *Rf1* allele was introduced from the radish to *B. napus* by cell fusion. As an X-irradiation technique was used to reduce the amount of transferred DNA, a limited amount of radish DNA was thought to be transferred into the *B. napus* nucleus. Our preliminary experiments suggest that plant KA11 in the kos CMS population may carry the *Rf2* allele (see Table 2, KA11×KC6 and KA2/KC1-1×KC6-4 as an example). During the cell fusion process and/or during the following three to six subsequent backcrosses, the *Rf2* gene might have been eliminated from the *B. napus* cybrid. However, we can not rule out the possibilities that unlinked *Rf1* and *Rf2* genes in radish were introduced to the same locus in *B. napus* or that the radish *Rf2* allele still remained in cybrid 18-3.

Pollen fertility in CMS *B. napus* cybrid 12-9 and alloplasmic ogu *B. napus* could not be restored by the *B. napus* Rf line. The alloplasmic ogu *B. napus* carries the complete mitochondrial genome from ogu radish. The 12-9 cybrid carries configurations of mtDNA different

from that of cybrid 18-3 (Sakai and Imamura 1993). The restriction digestion pattern of cybrid 12-9 shares more fragments with the kos radish than with *B. napus*, suggesting that the cybrid carries more radish mitochondrial genome than cybrid 18-3. A similar observation has been reported by Pelletier and Primard (1987). The intergeneric combination of nuclear and cytoplasmic genomes may cause alloplasmic dysfunction of the plant such as male sterility and aberrant flower morphology (Hakansson et al. 1993). Alloplasmic ogu *B. napus* and cybrid 12-9 may carry additional alloplasmic CMS-associated mitochondrial gene(s) of radish, and the gene(s) may prevent complete fertility restoration in *B. napus* even in the presence of the *Rf1* gene. In radish, comparison of a physical map of ogu and normal mtDNA revealed that the two cytoplasms are highly divergent, and rearrangements may have occurred during the evolution of the cytoplasms (Makaroff and Palmer 1988). Most examples of CMS-associated genes are chimeric genes that might have originated from a rearrangement of mtDNA during evolution of the species (Pring et al. 1993). Kos and ogu mitochondrial gene(s) may act as alloplasmic CMS gene(s) in certain nuclear genomes in radish, and the *Rf2* gene may alter the deleterious effects in the nuclear-cytoplasmic interaction in radish. If this is the case, the results obtained with *B. napus* indicate that *B. napus* does not have an endogenous *Rf2* allele and that the restoration of the fertility might be determined by additional alloplasmic mitochondrial gene(s) even with the presence of the *Rf1* gene. In any case, we have no clear evidence of *Rf2* function. A more detailed analysis of the genetics and function of the *Rf2* gene is necessary for elucidating the kos and ogu CMS-Rf systems.

Acknowledgements The authors thank Chie Tamura for her skillful technical assistance with Western blotting of *B. napus*. Also, for their collaboration we thank our colleagues at the Plantech Research Institute, especially Hideya Fujimoto, Yuske Kimura, Hiroaki Kurihara, Rieko Hakoda and Chie Tamura for their help with field investigations. The authors declare that the experiments performed comply with the current laws of Japan.

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