Theor Appl Genet (2005) 111: 1191–1200
DOI 10.1007/s00122-005-0052-x

ORIGINAL PAPER

**Seok-Hyeon Nahm · Hee-Jeong Lee · Shi-Woo Lee
Gyun-Young Joo · Chee-Hark Harn · Seung-Gyun Yang
Byung-Whan Min**

Development of a molecular marker specific to a novel CMS line in radish (*Raphanus sativus* L.)

Received: 31 January 2005 / Accepted: 16 July 2005 / Published online: 2 September 2005

Abstract In this study, we have investigated the cytoplasmic male sterility (CMS) of a novel male sterile radish line, designated NWB CMS. The NWB CMS was crossed with 16 fertile breeding lines, and all the progenies were completely male sterile. The degree of male sterility exhibited by NWB CMS is more than Ogura CMS from the *Cruciferae* family. The NWB CMS was found to induce 100% male sterility when crossed with all the tested breeding lines, whereas the Ogura CMS did not induce male sterility with any of the breeding lines. PCR analysis revealed that the molecular factor that influenced Ogura CMS, the *orf138* gene, was absent in the NWB CMS line, and that the *orf138* gene was not also expressed in this CMS line. In order to identify the cytoplasmic factors that confer male sterility in the NWB CMS line, we carried out RFLP analyses with 32 mitochondrial genes, all of which were used as probes. Fourteen genes exhibited polymorphisms between the NWB CMS line and other radish cultivars. Based on these RFLP data, intergenic primers were developed in order to amplify the intergenic regions between the polymorphic genes. Among these, a primer pair at the 3′ region of the *atp6* gene (5′-cgcttggactatgctatgtatga-3′) and the 5′ region of the *nad3* gene (5′-tcataga-gaaatccaatcgtcaa-3′) produced a 2 kbp DNA fragment as a result of PCR. This DNA fragment was found to be specific to NWB CMS and was not present in other CMS types. It appears that this fragment could be used as a DNA marker to select NWB CMS line in a radish-breeding program.

Communicated by I. Paran

S.-H. Nahm (✉) · H.-J. Lee · S.-W. Lee · G.-Y. Joo
C.-H. Harn · S.-G. Yang · B.-W. Min
Biotechnology Institute, Nong Woo Bio Co., Yeoju, Gyeonggi, South Korea
E-mail: shnahm1@nongwoobio.co.kr

Introduction

Cytoplasmic male sterility (CMS) is a maternally inherited trait, in which a plant is unable to produce functional pollen. CMS systems have been exploited for practical uses in the production of F1 hybrid seeds. CMS occurs in a variety of plant species and is often associated with novel mitochondrial open reading frames, which interfere with mitochondrial function and pollen development. CMS systems have been identified and characterized in a variety of plants, including arabidopsis, common bean, beet, maize, onion, petunia, rice, rye, sorghum, sunflower, and wheat (Schnable and Wise 1998). Several CMS systems have been characterized in the *Cruciferae* family: Polima, Napus, Ogura, and Anand CMS are well-characterized CMS systems belonging to the *Brassica* genus and Ogura and Kosena CMS are well-characterized CMS systems belonging to the *Raphanus* genus (Homme and Brown 1993, Homme et al.1997, Handa et al. 1995, Singh et al. 1996, Jean et al. 1997, Cardi and Earle 1997). The Ogura cytoplasm is one of the most extensively studied and is used for the production of hybrid seeds in radish. It is difficult to identify the CMS-related region or gene by the simple comparison of the genomic organizations of the Ogura CMS to the normal mitochondrial genomes, as the mitochondrial DNA of the Ogura cytoplasm exhibits a high degree of rearrangement as compared to that of a normal radish (Bonhomme et al. 1992, Makaroff and Palmer 1988, Makaroff et al. 1991, Krishnasamy and Makaroff 1993, 1994). However, a new open reading frame, which has been designated *orf138*, has been reported to be a crucial factor for Ogura CMS (Grelon et al. 1994). A more recent report indicates that the three regions of *orf138* perform important functions in transcript processing, as well as in the stability of *Brassica* cybrids (Bellaoui et al. 1997). In flowering plants, the suppression of the CMS phenotype by the nuclear restorer of fertility (*Rf*) genes is known as an example of nuclear-mitochondrial gene interaction. A single radish

nuclear gene, *Rfo*, restores the Ogura CMS in *Brassica napus* (Brown et al. 2003, Desloire 2003). This gene encodes a protein with multiple pentatricopeptide repeats, similar to the radish Kosena CMS restorer gene *orf687* and the *Petunia* CMS restorer gene *Rf2* (Koizuka et al. 2003, Bentolila et al. 2002).

The acquisition of a new CMS line and the introduction of its character into breeding programs are very important with regard to the development of commercial varieties. In this study, we have characterized a new CMS obtained from the novel male sterile radish line, NWB CMS, which was collected from South Korea. NWB CMS is unique and differs from other CMS types with regard to its degree of sterility and mitochondrial genome arrangement. We conducted RFLP analysis in order to identify a specific DNA marker that confers male sterility in the NWB CMS line.

Materials and methods

Plant materials

Table 1 lists the CMS lines and fertile lines utilized in this experiment. Seventeen *Raphanus* lines and cultivars, and six *Brassica* lines as references, were collected in South Korea, China, and from seed companies in other countries. These plants were then grown in a greenhouse prior to flowering. DNA was isolated for PCR and RFLP analysis, in order to identify the CMS type and to develop the NWB CMS specific DNA markers. Table 2 lists the 16 breeding lines used as male parents in order to test the induction of male sterility by several CMS lines. These breeding lines were pollinated onto the stigma of several CMS and F1 plants to obtain progeny and to compare the degree of male sterility occurring in their progenies. More than 20 progeny plants per cross were investigated with regard to male fertility.

Total DNA isolation

DNA extraction was performed, as previously described (Kang et al. 2001), with a few modifications. Young and healthy leaves were ground into a fine powder with liquid nitrogen. About 5 ml of frozen powder was aliquoted into pre-labeled 50 ml polypropylene tubes, which were chilled with liquid nitrogen. A preheated 20 ml extraction buffer (0.5 M NaCl, 100 mM Tris–HCl (pH 7.5), 50 mM EDTA (pH 8.0), 0.5% SDS) and 0.5 ml β-mercaptoethanol were then added to each tube. Each tube was mixed thoroughly with gentle agitation and incubated for 60 min at 65°C. A 24:1 ratio of chloroform to isoamyl alcohol was then added to the tubes and mixed thoroughly with gentle agitation. The tubes were then centrifuged for 15 min at 6,500 rpm. Supernatant DNA was precipitated by the addition of an equal volume of ice-cold isopropanol. The precipitated DNA was rinsed twice with 70% ethanol, then transferred to a sterile 1.5 ml microtube. The precipitated DNA was then dissolved in sterile water and quantified via agarose gel electrophoresis.

PCR-aided assay for CMS type identification

PCR was performed using primers, which were designed to be specific for the Ogura CMS specific *orf 138* region

Table 1 *Raphanus* and *Brassica* cultivars used for RFLP and PCR analysis

No.	line	Species	Fertility	Source[a]
1	NWB1	*Raphanus sativus*	Sterile	Korea collection
2	NWB2	*Raphanus sativus*	Sterile	Korea collection
3	BaekKwang	*Raphanus sativus*	Sterile	HeungNong seeds
4	SinJinJu	*Raphanus sativus*	Sterile	JungAng seeds
5	HaDong	*Raphanus sativus*	Sterile	HeungNong seeds
6	YR Takuyo	*Raphanus sativus*	Sterile	Japan collection
7	Kenka	*Raphanus sativus*	Sterile	Sakada seeds
8	Huyumine	*Raphanus sativus*	Sterile	Sakada seeds
9	MuCheong	*Raphanus sativus*	Sterile	China collection
10	ByeokOk	*Raphanus sativus*	Sterile	China collection
11	ChunJak	*Raphanus sativus*	Sterile	China collection
12	TaeBaek	*Raphanus sativus*	Sterile	China collection
13	HongPoong1	*Raphanus sativus*	Sterile	China collection
14	HongPoong3	*Raphanus sativus*	Sterile	China collection
15	WonBaek	*Raphanus sativus*	Sterile	China collection
16	NWMF1	*Raphanus sativus*	Fertile	NongWooBio seeds
17	NWMF2	*Raphanus sativus*	Fertile	NongWooBio seeds
18	Komatsuna	*Brassica rapa*	Sterile	SunCheon Uni. Korea
19	Donskyaa	*Brassica juncea*	Sterile	SunCheon Uni. Korea
20	Jasai	*Brassica juncea*	Sterile	SunCheon Uni. Korea
21	Anand	*Brassica rapa*	Sterile	KangWon Uni. Korea
22	NokPoong	*Brassica rapa*	Fertile	China collection
23	ChuKang	*Brassica rapa*	Fertile	China collection

[a]Each line was collected from several countries, seed companies, or universities

Table 2 Results of the test crosses to analyze the rate of CMS induction in the progenies from NWB CMS and three other Ogura CMS lines

Breeding lines[a]	NWB1 (NWB CMS)	BaekKwang (Ogura CMS)	Kenka (Ogura CMS)	Huyumine (Ogura CMS)
SB1523	Sterile	Sterile	Sterile	Sterile
SB1978	Sterile	Sterile	Sterile	Sterile
CT1642	Sterile	Sterile	Sterile	Sterile
JD0340	Sterile	Sterile	Sterile	Sterile
SM0462	Sterile	Sterile	Sterile	Sterile
SHW52	Sterile	Sterile	Sterile	Sterile
CT1968	Sterile	Sterile	Sterile	Sterile
CT2015	Sterile	Sterile	Sterile	Sterile
1934	Sterile	Fertile	Fertile	Fertile
Mn002	Sterile	Fertile	Fertile	Sterile
Mn003	Sterile	Fertile	Seg[b] (8:12)	Fertile
YB-1	Sterile	Fertile	Fertile	Fertile
1825	Sterile	Fertile	Seg[b] (9:15)	Seg[b] (10:12)
AD1002	Sterile	Sterile	Sterile	Fertile
Mn006	Sterile	Sterile	Seg[b] (9:13)	Sterile
SG1634	Sterile	Fertile	Fertile	Fertile

[a]Sixteen breeding lines were used as male parents and four CMS lines were used as female parents
[b]Segregation of male sterile and male fertile plants

(Yamagishi and Terachi 1996), 5′-gacatctagagaagttaa-aaat-3′ and 5′-agcaattgggttcacaaagcat-3′. PCR with these primer pairs resulted in the amplification of a 278 bp DNA fragment in plants with radish Ogura-type CMS and a 239 bp fragment in plants with radish Kosena-type CMS (Yamagishi and Terachi 1996, Iwabuchi et al. 1999) and generated no products in male fertile plants. These primer pairs were utilized to determine whether the NWB CMS line is a previously known CMS type, or a novel CMS type, as was previously described by Yamagishi and Terachi (1996).

Total RNA isolation and Northern analysis

Total RNA was isolated from flower buds just before bursting, using Trizol (GIBCO BRL) reagent. The RNA was fractionated on 1.2% agarose gel containing 0.22 M formaldehyde at 30 V overnight, transferred to a nylon membrane, then fixed by UV cross-linking. The filter was hybridized at 65°C for 16 h with a radioactively labeled probe in hybridization solution (5× SSC, 0.5% SDS, 5× Denhardt's reagent), washed initially with 2× SSC, 0.1% SDS for 30 min at room temperature, then with 0.5× SSC, 0.1% SDS for 60 min at 60°C. The probes were prepared via PCR amplification from the "BaekKwang" Ogura CMS line as a DNA template, using primer pairs specifically designed to amplify the *orf138* and *rrn18* genes. The *orf138* gene was amplified via PCR, as described previously (Yamagishi and Terachi 1996). After the washing step, the filters were placed under Agfa CP-BU film (European Communities) for 3 days.

RFLP analysis of mitochondrial DNA

RFLP analysis was performed, as previously described (Kang et al. 2001), with some minor modifications. DNA was extracted from two of the NWB CMS lines, two of the Ogura CMS types, and two of the male fertile lines, all within the seedling stage. The RFLP survey filters were then prepared from DNAs which were digested with six different restriction enzymes: *Bam*HI, *Eco*RI, *Dra*I, *Eco*RV, *Hin*dIII, and *Xba*I. Restriction digestion was carried out with 1 U restriction enzymes per microgram of DNA. Approximately 10 μg of radish total DNA was then loaded and separated on 0.8% agarose gel in a 0.5× TAE buffer for 12 h at 50 V (6 V/cm). Using PCR primers, which were designed based on the *Arabidopsis* mitochondrial genome sequence (Unseld 1997), 32 radish mitochondrial genes (Table 3) were amplified and then used as probes for RFLP analysis. The PCR amplification of mitochondrial genes was conducted with the total DNA of the radish NWB CMS1 line as a DNA template, except for the *orf224* gene, which was amplified with the *Arabidopsis thaliana* ecotype Columbia DNA as a template. In an Eppendorf Mastercycler, 35 PCR cycles were conducted. Each cycle consisted of 30 s at 94°C, 30 s at 54°C, and 1 min at 72°C. The probes were labeled with α-[^{32}P]dCTP, using the random hexamer procedure (Promega, Madison, USA). The labeled probes were denatured via alkali treatment with a final concentration of 0.3 N NaOH, then added to filters in 40 ml of hybridization buffer (5× SSC, 0.5% SDS, 5× Denhardt's reagent). Hybridization was conducted at 65°C for 24 h. Filters were washed with 1× SSC, 0.1% SDS for 60 min, then with 0.5× SSC, 0.1% SDS for an additional 60 min. The filters were then placed under Agfa CP-BU film (European Communities) for several days, depending on the strength of the signals.

NWB CMS detection using specific PCR primers

For the rapid and reliable identification of the CMS type, we developed a PCR marker system, which was able to specifically detect the novel NWB CMS line.

Table 3 Breeding lines used for test cross to analyze the rate of CMS restoration in the progenies from NWB CMS lines and to investigate the possibilities as maintainer lines for F1 seed production

No.	Breeding lines[a]	Origin	Male fertility in progeny	No.	Breeding lines[a]	Origin	Male fertility in progeny
1	KUS1	Korea	Sterile	30	OSS1	Japan	Sterile
2	NKS1	Japan	Sterile	31	PUCHS	India	Sterile
3	MSAS1	Japan	Sterile	32	TSWES	India	Sterile
4	MKS1	Japan	Sterile	33	KJS1	Japan	Sterile
5	MYS1	Japan	Sterile	34	KCS1	Japan	Sterile
6	MIS1	Japan	Sterile	35	NBJS1	China	Sterile
7	MCS1	Japan	Sterile	36	NBCTS1	Japan	Sterile
8	MKS2	Japan	Sterile	37	BD13S1	China	Sterile
9	MCS2	Japan	Sterile	38	BAS1	Korea	Sterile
10	BNS1	Korea	Sterile	39	4JSS1	Japan	Sterile
11	SUS1	Korea	Sterile	40	SGS1	China	Sterile
12	SYAS1	Japan, Korea	Sterile	41	SEOS1	Korea	Sterile
13	SCS11	Japan, Korea	Sterile	42	SESBS1	Korea	Sterile
14	AKS1	Korea, Japan	Sterile	43	SHWS	Japan	Sterile
15	ABS1	Korea	Sterile	44	SJMTS1	Korea	Sterile
16	AB20S1	Korea, Europe	Sterile	45	SMDS1	Korea	Sterile
17	ASS1	Korea	Sterile	46	ATR1	Korea	Sterile
18	ACS1	Korea, Japan	Sterile	47	YHS1	Korea	Sterile
19	WJS1	Korea	Sterile	48	USJS1	Korea	Sterile
20	UYS1	Korea	Sterile	49	WG108S1	Korea	Sterile
21	UCS1	Korea, Japan	Sterile	50	OWCS1	Korea	Sterile
22	ISS1	Japan	Sterile	51	USCS11	Korea	Sterile
23	CCSS1	China, Japan	Sterile	52	RR20S1	Europe	Sterile
24	JKS1	Korea, Japan	Sterile	53	WONBS1	China	Sterile
25	CDS1	Japan	Sterile	54	JJDS1	Korea	Sterile
26	HSD13S1	China	Sterile	55	CTS1	Japan	Sterile
27	HSYS1	China, Korea	Sterile	56	SUMS1	Japan	Sterile
28	60WS1	China	Sterile	57	REDS1	China	Sterile
29	OHS1	Japan	Sterile	58	H3KJS1	China, Japan	Sterile

Each line was used as a male parent and NWB CMS line as a female parent. Each line consisted of two or three sub-breeding lines, so more than 58 lines were investigated for CMS restorer or maintainer line. More than 20 progeny plants per cross were investigated for male fertility

[a]Breeding lines were developed from several cultivars and wild radishes collected from several countries. Some lines were developed from two cultivars collected from different countries

Based on RFLP analysis, it was known that *atp6* and *nad3* genes were located in regions of about 6 kbp in the NWB CMS line, and not in the Ogura CMS and male fertile lines. Therefore, we designed primer pair at the 3′ region of the *atp6* (5′-cgcttggactatgctatgtatga-3′) and the 5′ region of the *nad3* (5′-tcatagagaaatccaatcgtcaa-3′) gene. This primer pair was utilized for PCR amplification to determine its applicability to the detection of the NWB CMS line. In an Eppendorf Mastercycler, 35 PCR cycles were conducted. Each cycle consisted of 30 s at 94°C, 30 s at 54°C, 1 min at 72°C, and then the PCR products were analyzed via agarose gel electrophoresis.

Results

Comparison of male sterility between NWB and Ogura CMS

Ogura CMS lines exhibited rudimentary, gloomy, shrunken anthers, with no visible pollen, while the anthers of the NWB CMS lines were more yellowish, with some visible pollen. These phenotypes were more advantageous for attraction of insect pollinator honeybee and seed setting. The amount of pollen per anther in the NWB CMS line was approximately half that of male fertile lines, but all the pollen were unviable. In order to compare the degrees of male sterility induction between the NWB CMS and Ogura CMS lines, one NWB and three Ogura CMS lines were used as female parents for cross with of 16 male fertile breeding lines. The progenies of all 16 lines were found to be completely male sterile when crossed with the NWB CMS line (Table 2). When we backcrossed NWB CMS line-derived male sterile F1 plants with the 16 male fertile breeding lines, all the progenies were male sterile (data not shown), independent of their male parents, thereby indicating that the CMS phenomenon was induced by cytoplasmic factors, including mitochondrial gene(s). However, when the 16 lines were crossed with the Ogura CMS lines, the progenies of 9–10 of the breeding lines were observed to become male sterile and the others became either fertile or segregated (Table 2). The breeding lines utilized in this experiment were fixed lines, which were self-pollinated more than six times. Therefore, the appearance of segregating progenies after crossing with Ogura CMS is quite unusual. There is also a possibility of the presence of heterozygous plants in the test cross experiment, due to the incomplete homozygosity associated with insufficient self-pollinated generations. Ogura CMS lines used

in this study showed different patterns of fertility restoration by 16 different breeding lines used as male parents. Ogura CMS was classified into nine types based on the *orf138* gene sequence from 107 Japanese wild radishes, 29 cultivars, and 7 *Raphanus raphanisturum* (Yamagishi 2001). Although it is not verified that these nine types of Ogura CMS are different in fertility restoration mechanism, it is also possible that the altered *orf138* gene sequence may be related to the difference in restorer line. The difference in fertility restoration from the three Ogura CMS lines used in our test cross experiment may also be related to the altered *orf138* sequence, though DNA sequences of *orf138* gene were not compared. On the other hand, fertility restoration by mitochondrial-nuclear genome interaction may be more complicated than we assumed.

Male fertility in Ogura CMS plants can be restored by the control of the nuclear restorer gene *Rfo*, which regulates the expression of the *orf138* gene. In order to determine whether male fertility can be restored in the NWB CMS system, the NWB CMS line was crossed with 58 breeding lines, which were bred from cultivars and wild radishes collected from Korea, Japan, China, India, and Europe (Table 3). We failed to detect any restorer line, and all the progenies of the cross-pollinations became male sterile, indicating that the male sterility of NWB CMS might constitute a cytoplasmic trait. It is also possible that there will be restorer genes from wild radish and cultivars not tested in this study and male sterility is controlled by the action of nuclear genes in this novel NWB CMS system.

Molecular identification of NWB CMS

In order to determine whether the NWB CMS type differs from the Ogura CMS type by virtue of the other factor(s) influencing the CMS phenomenon, we attempted to detect the presence of the *orf138* gene, the specific factor for Ogura CMS, in the NWB CMS line. Using PCR, we determined that the Ogura *orf138* gene-specific primer pair results in the amplification of a 278 bp DNA fragment in the Ogura-type CMS radish plants, but no PCR products were detected in the NWB CMS and male fertile plants (Fig. 1). RFLP and Northern analyses between the NWB CMS lines and other radish cultivars were performed using the Ogura CMS specific *orf138* gene as a probe, which was prepared by PCR amplification from the Ogura CMS line "BaekKwang" as a DNA template, using primer pairs specifically designed to amplify the *orf138* gene (Yamagishi and Terachi 1996). Figure 2a illustrates an RFLP pattern of total DNA, digested by *Hin*dIII. The *orf138* gene was detected only in the Ogura CMS line (3.8 kbp), but the *orf138* homologous DNA fragment was also detected in all lines, at approximately 6.0 kbp. Similar band patterns were also detected when other restriction enzymes were used for RFLP analysis. When RFLP analysis was conducted with the *Bam*HI and *Eco*RI restriction enzymes, the *orf138*-specific fragments were detected at approximately 2 and 10 kbp fragments, respectively, in the Ogura CMS line (Fig. 2b, c). However, weaker signals were also detected, at approximately 4 kbp, in all lines, when the *Bam*HI and *Eco*RI restriction enzymes were used. When membrane washing was conducted under more stringent conditions (0.1× SSC, 0.1% SDS for 60 min at 60°C), the signal became weaker, but did not disappear completely. These results indicate that the radish mitochondria genome may contain a small DNA sequence homologue of the *orf138* gene, regardless of CMS phenotype. The first six Ogura CMS lines (lanes 1–6 in Fig. 2b, c) were hybridized with the Ogura CMS specific *orf138* gene, but other CMS types were not hybridized. These Ogura CMS lines may have a different CMS mechanism, as compared to the NWB CMS line. Another CMS line "WonBaek" (lane 9 in Fig. 2b, c), collected from China, also resulted in no PCR products using this Ogura-specific primer, and this line may also have a different CMS mechanism compared to Ogura CMS.

Development of novel NWB CMS specific markers

In order to establish a DNA marker for the accurate identification of the CMS type in our breeding program and for the detection of seed contamination in terms of quality control, RFLP analysis was conducted, and

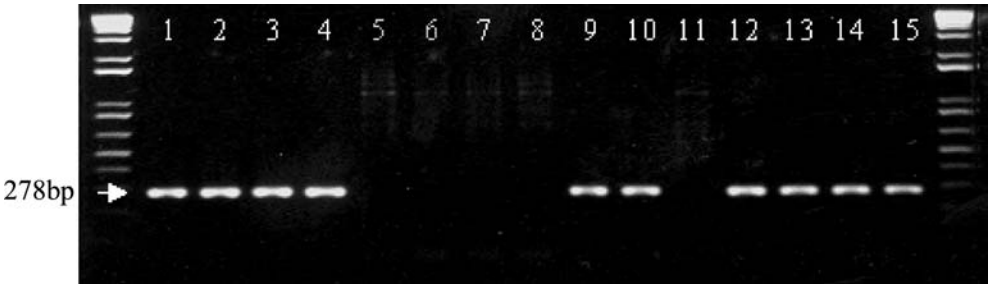

Fig. 1 Amplification of DNA fragments using primer pairs designed at the Ogura CMS specific *orf138* region (Yamagishi and Terachi 1996) in several radish lines, for the characterization of NWB CMS line. *Lane 1* BaekKwang (Ogura CMS), *lane 2* SinJinJu (Ogura CMS), *lane 3* YR Takuyo (Ogura CMS), *lane 4* Kenka (Ogura CMS), *lane 5* NWB CMS1, *lane 6* NWB CMS2, *lane 7* NWMF1 (fertile), *lane 8* NWMF2 (fertile), *lane 9* MuCheong (unknown CMS), *lane 10* ByeokOk (unknown CMS), *lane 11* WonBaek (unknown CMS), *lane 12* ChunJak (unknown CMS), *lane 13* TaeBaek (unknown CMS), *lane 14* HongPoong1 (unknown CMS), *lane 15* HongPoong3 (unknown CMS)

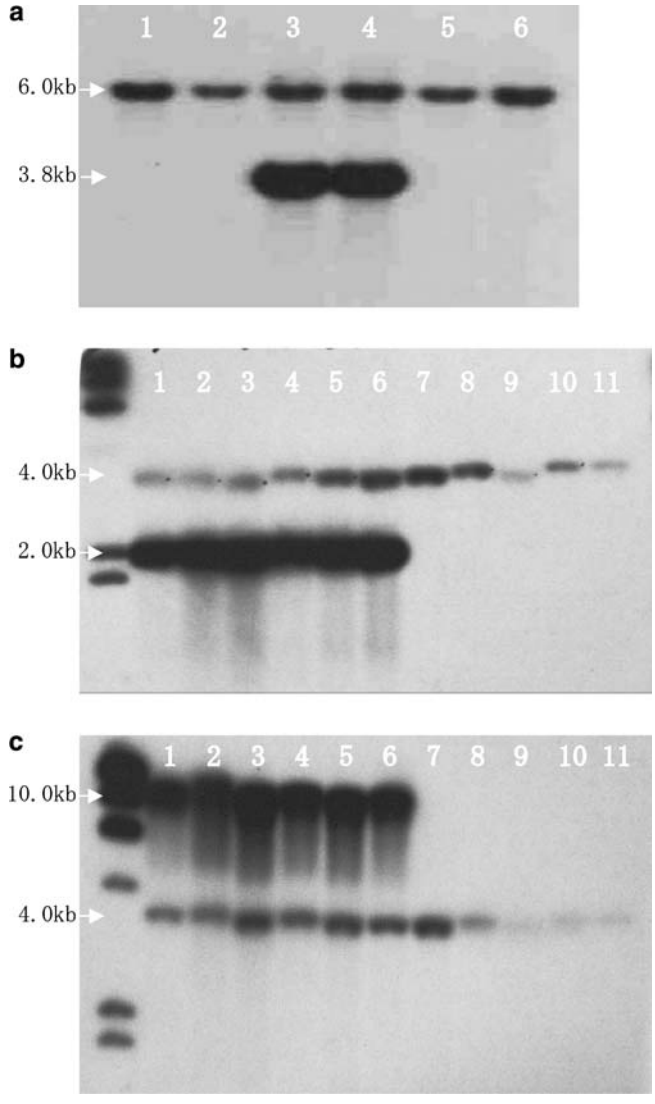

Fig. 2 RFLP analyses between the NWB CMS line and other radish cultivars. Total DNAs were digested with *Hin*dIII (**a**), *Bam*HI (**b**), and *Eco*RI (**c**), blotted onto a nylon membrane and then hybridized with the Ogura CMS specific *orf138* gene as probes. **a** *Lane 1* NWB CMS1, *lane 2* NWB CMS2, *lane 3* BaekKwang (Ogura CMS), *lane 4* SinJinJu (Ogura CMS), *lane 5* NWMF1 (male fertile), *lane 6* NWMF2 (male fertile). **b** and **c** *Lane 1* BaekKwang, *lane 2* SinJinJu, *lane 3* HaDong, *lane 4* YR Takuyo, *lane 5* Kenka, *lane 6* Huyumine, *lane 7* NWB CMS1, *lane 8* NWB CMS2, *lane 9* WonBaek, *lane 10* NWMF1, *lane 11* NWMF2

polymorphic DNA patterns were observed between the NWB CMS line and other radish lines. A total of 32 radish mitochondrial genes (Table 4) were assayed with regard to polymorphisms between NWB CMS lines and other radish lines. Out of 32 mitochondrial genes, 14 genes exhibited polymorphisms with at least one restriction enzyme between the NWB CMS line and the other radish lines (Table 5). These results indicate that the mitochondrial genome of NWB CMS exhibited marked rearrangement compared to that of the Ogura CMS line and a normal radish. RFLP analysis revealed that a 6.0 kbp fragment had been generated by the *atp6* and *nad3* genes in the NWB CMS lines, when they were digested with the *Eco*RI restriction enzymes and 15 kbp with *Eco*RV restriction enzymes (Fig. 3). However, different band patterns were exhibited in the Ogura CMS line and the male fertile radish line. These results indicate that the *atp6* and *nad3* genes are located in a 6.0 kbp mitochondrial DNA fragment in the NWB CMS line, but not in the Ogura CMS line and male fertile radish lines. Therefore, we designed several primer pairs nested in the *atp6* and *nad3* genes, in order to amplify this region, in the hopes of determining which mitochondrial factors influence the CMS phenomenon. One of these primers, the 3′ region of the *atp6* (5′-cgcttggactatgctatgtatga-3′) gene and the 5′ region of the *nad3* (5′-tcatagagaaatccaatcgtcaa-3′) gene, which commonly amplified a DNA fragment of about 2.0 kbp, harbored a portion of the *atp6* and *nad3* genes specifically in the radish NWB CMS line, but not in the other radish and *Brassica* CMS lines (Fig. 4). *Brassica* CMS and fertile lines were investigated in this experiment to compare the mitochondrial genome configuration in *atp6* and *nad3* intergenic region between radish NWB CMS and Brassica lines. Therefore, the 2.0 kbp DNA fragment obtained with this primer set constituted a DNA marker for the identification of the NWB CMS type.

Northern blot analysis of mitochondrial genes

Since polymorphic bands were determined to be present in NWB CMS and other CMS types near the *atp6*, *nad3*, and *orf138* genes, the expression levels or transcript sizes of these genes were investigated. Northern blot analysis revealed that the *orf138* gene is expressed specifically in the Ogura CMS line, but not in the NWB CMS line and the male fertile lines (Fig. 5a). However, the *atp6* and *nad3* genes were expressed in all tested CMS lines (Fig. 5b, c). The *atp6* gene expressed three fragments at approximately 1.2, 1.4, and 1.6 kbp regions in Ogura CMS and one fragment at the 1.0 kbp region in the NWB CMS lines and male fertile lines. These results are consistent with previous reports (Makaroff 1989) that the Ogura *atp6* gene was co-transcribed with a novel *orf*105 gene, which was not observed in the normal male fertile line. The *nad3* gene encoding the NADH dehydrogenase subunit 3 in the Ogura CMS radish expressed one fragment at an approximately 1.4 kbp region, although the original transcript size of the *nad3* gene was expected to be ~400 bp. This is attributable to the possibility that the *nad3* gene is co-transcribed with the *rps12* gene, which encodes mitochondria ribosomal protein S12 (Rankin 1996).

Discussion

NWB CMS is a novel genetic source for CMS, which was screened several years ago by breeders at the Nong Woo Bio Co. In order to use this CMS line routinely for

Table 4 Mitochondrial genes, PCR primers, and product size for RFLP analysis

Gene	Gene product	PCR primer sequence (5' → 3')		Size (bp)
atp1	ATP synthase subunit alpha	F: gaaagcatctggttccattt	R: agagctgcggaactaacgaa	1,479
atp6	ATP synthase subunit 6	F: actaaaaagggaggaggaaaa	R: atctcattcatacatagcatagt	485
apt9	ATP synthase subunit 9	F: ccgagatgttagaaggtgcaa	R: atcaaaaaggccatcattgg	214
cox1	Cytochrome c oxidase sub 1	F: ttcgtctccttgatagctgga	R: atctggttcgatggctgttc	1,561
cox2	Cytochrome c oxidase sub 2	F: cgtaaaggcatgattagttcca	R: tcacaatttctccttgtgatgc	674
cox3	Cytochrome c oxidase sub 3	F: caagtccatggcctatttcg	R: tcatatacctccccaccaat	758
ccb203	Cytochrome c biogenesis orf203	F: gagccgagtgacgtagatgc	R: cgtgtcgttcgtaatggaaa	547
ccb206	Cytochrome c biogenesis orf206	F: tgatcttctcctccacacca	R: cgagaccgaaattggaaaaa	569
ccb256	Cytochrome c biogenesis orf256	F: gttgggcgctttaaccatt	R: agctacgcgcaaattctcat	719
ccb382	Cytochrome c biogenesis orf382	F: ctgcccagaacgaagagaag	R: ttcgttatttccgggtcttt	1,149
ccb452	Cytochrome c biogenesis orf452	F: cgacagaagaacacccaaca	R: ttttatggtcgtgccttgtg	745
cob	Apocytochrome B	F: aaggaaccaacgattctctcttc	R: tgtgatcagtctcatccgtgt	1,169
nad1	NADH dehydrogenase sub 1	F: ccgacccgctataataattcc	R: tgttccagctgaaatacttgga	367
nad2	NADH dehydrogenase sub 2	F: ccgaaaccaaggggatacta	R: ggaaggttgccacgaataag	1,626
nad3	NADH dehydrogenase sub 3	F: gcacccctttccattcata	R: gatgtcagaatttgcaccaa	342
nad4	NADH dehydrogenase sub 4	F: acagggaattggaggtagca	R: tgccatttgaatcggagaat	380
nad4L	NADH dehydrogenase sub 4L	F: tacctcggactcggaagtt	R: ttcggggaatcctccttaat	213
nad5	NADH dehydrogenase sub 5	F: caatcgtcggaatgtgtacg	R: ccaatttttgggccaattc	359
nad6	NADH dehydrogenase sub 6	F: gtgagtgggtcagtcgtcct	R: cctgctttggtctctggttt	584
nad7	NADH dehydrogenase sub 7	F: tctatgatggcccaagaaca	R: acaccactgaatccccaatc	446
nad9	NADH dehydrogenase sub 9	F: ttatccgtcgctacgctgtt	R: cccaagaaatgggtcaaaaa	531
matR	Maturase	F: gcttcccaagctctatgctgt	R: actgcttgccacctacgc	751
orfB	ATP synthase subunit 8	F: tgcctcaactggataaattcac	R: ttccttggccatgtacaaca	465
orfX	Unknown protein	F: gcatcggaaacgattctagg	R: ttactttgccaggttctaggg	797
orf240	Unknown protein	F: cttctgacctcccagtccag	R: tgaggggaaggttggtcata	570
rpl2	Ribosomal protein L2	F: tgctgcgaagaattgatctg	R: acattgcttaggaccaacgg	827
rpl16	Ribosomal protein L16	F: tgacataagattctcggccc	R: gggagacgtgctatatccga	486
rps3	Ribosomal protein S3	F: tcggatatagcacgtctccc	R: tacgcgactcaccttcggct	1,513
rps4	Ribosomal protein S4	F: ccgaagaggaaggtttggat	R: ccgaagattgaggaacagga	934
rrn5	5S ribosomal RNA protein	F: ttatttcttcaccgggcttg	R: gagacgtgaaaacacccgat	108
rrn18	18S ribosomal RNA protein	F: gattcaatccagccacaggt	R: catgcaagtcgaacgttgtt	1,795
rrn26	26S ribosomal RNA protein	F: tctcccttaacaccaacgg	R: atgacttgtggctaggggtg	1,890

the production of hybrid seeds in the radish-breeding program, we attempted to determine the male sterility inheritance of the NWB CMS line. In this report, the NWB CMS line was determined to be different in several aspects from any of the other CMS types reported.

1. The degree of sterility of the NWB CMS was more profound than any of the other CMS types, including Ogura CMS, which had previously been well characterized (Table 1). Indeed, the NWB CMS line induced complete sterility in progenies, when crossed with any of the tested breeding lines. Even when the F1 plants of the NWB CMS and breeding lines were backcrossed to the male fertile breeding lines, the progenies became male sterile, independent of their male parents (data not shown), indicating that the CMS phenomenon of the NWB CMS line may be induced by cytoplasmic factor(s).
2. The NWB CMS line was not determined to contain the Ogura CMS specific factor, *orf138* (Figs. 1, 2).
3. There is a polymorphism of mitochondrial genome between NWB CMS and Ogura CMS. RFLP analysis revealed that 14 mitochondrial genes showed (Table 5) different band patterns between NWB CMS and Ogura CMS.
4. The region between the *atp6* and *nad3* genes in the NWB CMS line harbored a PCR-based DNA marker, which proved useful in the identification of the NWB CMS type specifically, but not the other types of CMS, including the Ogura and Anand CMS types (Fig. 4).
5. The Ogura CMS line was restored to male fertility when crossed with several breeding lines, whereas the NWB CMS line was not restored to male fertility, and all the progenies became male sterile (Tables 2, 3). When all data are taken together, the NWB CMS constitutes a novel CMS type and an important genetic source, which can be utilized extensively in a radish-breeding program.

Since the *atp6* and *nad3* genes exhibited a high degree of rearrangement in the NWB CMS line, based on RFLP analysis (Table 5), one of the major questions which has been raised is whether or not the regions of the *atp6* and *nad3* genes exhibit functional roles in the maintenance of the CMS phenotype. DNA sequence analysis revealed that the *nad3* gene is located approximately 1.4 kbp downstream of the *atp6* gene (data not shown). However, the DNA sequences of each gene in the NWB CMS line and the normal male fertile radish were identical (data not shown). In addition, both the *atp6* and *nad3* genes were expressed in the NWB CMS, Ogura CMS, and male fertile lines, when hybridized with an intergenic DNA fragment as a probe (Fig. 5).

Table 5 Mitochondrial genes showed polymorphisms and polymorphic fragments' size difference between radish NWB CMS, Ogura CMS, and male fertile lines

Gene	Restriction enzyme	Restriction fragment size (kbp)		
		NWB CMS line	Ogura CMS line	Male fertile 1/2[a]
atp1	*Hin*dIII	7.0	6.5	6.5/6.5
atp1	*Xba*I	10.0	9.0	12.0/9.0
atp6	*Bam*HI	13.0	3.5	23.0/20.0
atp6	*Dra*I	23.0	6.6	20.0/8.0
atp6	*Eco*RI	6.0	6.6	12.0/9.0
atp6	*Eco*RV	15.0	4.4	6.6/6.6
atp6	*Xba*I	6.6	3.5	9.0/9.0
apt9[b]	*Dra*I	a: 7.0	a: 23.0	a: 23.0/23.0
		b: 1.5	b: 20.0	b: 1.5/2.2
apt9[b]	*Eco*RI	a: 6.6	a: 6.0	a: 10.0/10.0
		b: 4.0	b: 3.8	b: 3.5/3.8
apt9[b]	*Eco*RV	a: 6.4	a: 15.0	a: 10.0/8.0
		b: 5.5	b: 6.0	b: 1.5/5.0
apt9[b]	*Hin*dIII	a: 2.0	a: 4.0	a: 3.8/5.0
		b: 1.8	b: 3.5	b: 1.8/1.0
cox1	*Xba*I	10.0	15.0	15.0/15.0
nad1	*Xba*I	10.0	15.0	15.0/15.0
nad3	*Eco*RI	6.0	6.6	12.0/9.0
nad3	*Hin*dIII	4.4	5.0	6.0/6.0
nad4L	*Dra*I	6.6	12.0	12.0/12.0
matR	*Eco*RI	6.0	4.2	4.2/9.0
orfB	*Hin*dIII	3.7	3.5	3.9/4.6
orfB	*Xba*I	9.0	6.6	15.0/6.0
rpl2	*Xba*I	6.0	6.6	8.0/3.6
rpl16	*Dra*I	6.0	9.0	9.0/9.0
rps3	*Dra*I	6.6	7.5	7.5/7.5
rrn5	*Eco*RV	9.0	10.0	10.0/10.0
rrn5	*Xba*I	15.0	10.0	6.0/10.0
rrn18	*Eco*RI	9.0	2.0	2.0/2.0
rrn18	*Xba*I	15.0	10.0	10.0/10.0

[a]Two male fertile lines NWMF1 and NWMF2 showed different RFLP patterns when some genes were used as probes. New CMS line "WonBaek" shows the same RFLP pattern with NWMF2

[b]*atp9* gene is present as two copies in radish lines used in this study as previously published (Albaum et al. 1995), so polymorphic fragments were differentiated by characters 'a' and 'b'

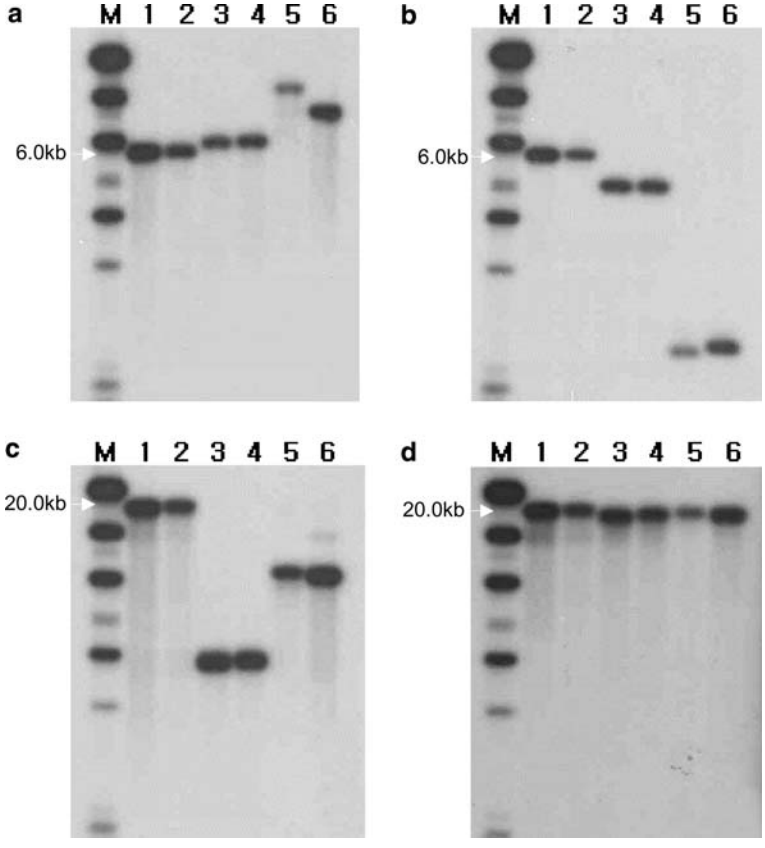

Fig. 3 RFLP analyses between the NWB CMS lines and other radish cultivars. Total DNAs of several radish cultivars were digested with *Eco*RI (**a**, **b**) and *Eco*RV (**c**, **d**), blotted onto a nylon membrane, then hybridized with the mitochondrial genes *atp6* (**a**, **c**) and *nad3* (**b**, **d**) used as probes. *Lane M* λ *Hin*dIII size marker. *lane 1* NWB CMS1, *lane 2* NWB CMS2, *lane 3* BaekKwang (Ogura CMS), *lane 4* SinJinJu (Ogura CMS), *lane 5* NWMF1 (male fertile), *lane 6* NWMF2 (male fertile)

Fig. 4 PCR amplification of the intergenic region between the *atp6* and *nad3* genes using primer pairs designed for each gene's internal regions. *Lane 1* NWB CMS1, *lane 2* NWB CMS2, *lane 3* BaekKwang (Ogura CMS), *lane 4* SinJinJu (Ogura CMS), *lane 5* HaDong, *lane 6* YR Takuyo (Ogura CMS), *lane 7* Kenka (Ogura CMS), *lane 8* Huyumine, *lane 9* MuCheong (unknown CMS), *lane 10* ByeokOk (unknown CMS), *lane 11* ChunJak (unknown CMS), *lane 12* TaeBaek (unknown CMS), *lane 13* HongPoong1 (unknown CMS), *lane 14* HongPoong3 (unknown CMS), *lane 15* WonBaek (unknown CMS), *lane 16* NWMF1 (male fertile), *lane 17* NWMF2 (male fertile), *lane 18* Komatsuna, *lane 19* Donskyaa, *lane 20* Jasai, *lane 21* Anand, *lane 22* NokPoong, *lane 23* ChuKang

Therefore, it appears that the *atp6* and *nad3* regions are unrelated to the CMS phenotype. Rather, another region of the mitochondrial genome may exert a crucial influence on sterility in the NWB CMS line. It is necessary to test the other 12 genes in more detail, in order to determine mitochondrial factors related to the CMS phenotype.

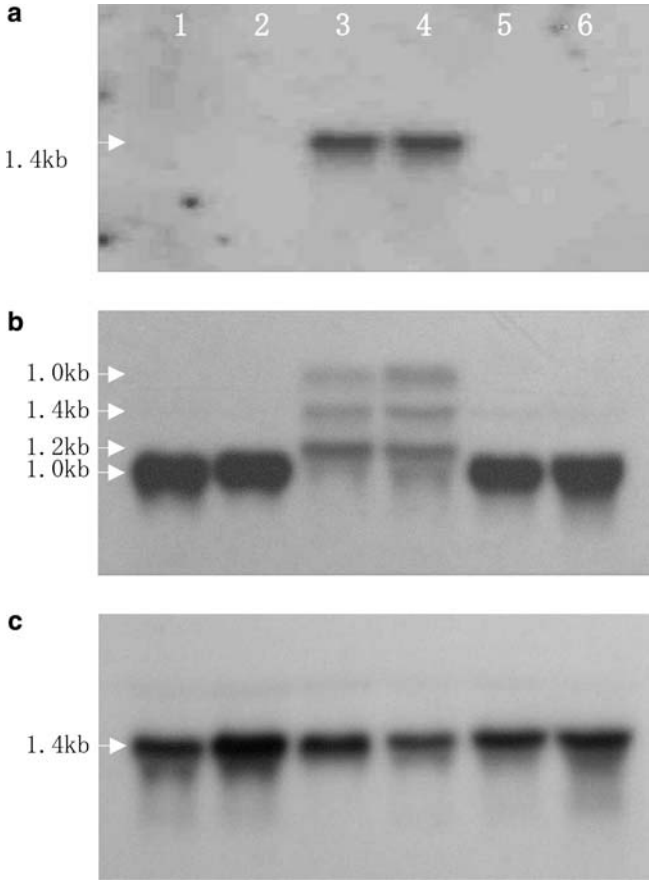

Fig. 5 Northern analyses between NWB CMS lines and other radish cultivars. Total RNAs from flower buds just before bursting were fractionated in a 1.2% agarose gel containing 0.22 M formaldehyde, transferred to a nylon membrane, and then the filters were hybridized with a radioactively labeled *orf138* (**a**), *atp6* (**b**), and *nad3* (**c**) genes. *Lane 1* NWB CMS1, *lane 2* NWB CMS2, *lane 3* BaekKwang (Ogura CMS), *lane 4* SinJinJu (Ogura CMS), *lane 5* NWMF1 (male fertile), *lane 6* NWMF2 (male fertile)

The "WonBaek" CMS line collected from China would constitute another CMS line, which is different from Ogura CMS. The "WonBaek" CMS genome did not produced the *orf138* gene-specific PCR product, using an Ogura CMS specific primer (Fig. 1). RFLP analyses also demonstrated that the *orf138* gene is not present in the WonBaek CMS (Fig. 2). RFLP analysis between the "WonBaek" CMS line and the other CMS line also showed high polymorphism (data not shown). Ten genes, out of the 32 mitochondrial genes used as probes, showed polymorphisms between the "WonBaek" and Ogura CMS lines. Also, 12 genes, out of 32 mitochondrial genes used as probes, showed polymorphisms between the "WonBaek" and NWB CMS lines. But, when compared with RFLP of male fertile lines, "WonBaek" showed the same band pattern with the NWMF2 line (Table 5). These results imply that most part of "WonBaek" mitochondrial genome is similar to the male fertile line NWMF2. NWB CMS specific primer pairs did not amplify when "WonBaek" was used as PCR template (Fig. 4, line 15). Therefore, similar to the NWB CMS line, the WonBaek CMS line may also constitute another distinct CMS line

The Ogura CMS specific primer was also determined to amplify a 239 bp fragment from the Kosena CMS line, which is controlled by the *orf125* gene homologous to the *orf138* sequence, except for two amino acid substitutions and a 39 bp deletion in the *orf138* coding sequence (Yamagishi 1996). These results indicate that the crucial factors that determine male sterility for the Ogura and Kosena CMS, NWB CMS, and WonBaek CMS lines would be different.

In general, the nuclear restorer genes that specifically suppress the CMS phenotype tend to modify the expression of CMS-associated regions or genes, but do not modify the expression of other mitochondrial genes (Jean et al. 1997). The Ogura CMS restorer gene *Rfo*, the Kosena CMS restorer gene *orf687*, and the Petunia CMS restorer gene *Rf2* (Koizuka et al. 2003, Bentolila et al. 2002), all encode the same polypeptide. Therefore, similar proteins could theoretically control fertility restoration in several different CMS systems. The mecha-

nism underlying the restoration of fertility remains unclear, but the combination of the activity of nuclear and cytoplasmic factors would constitute a controlling factor with regard to male sterility and fertility restoration in the radish Ogura CMS line (Hanson and Bentolia 2004). As shown in Table 3, the screening of a male fertility line was not successful by the crossing of the NWB CMS to the 58 breeding lines. The finding of a restorer line and gene against NWB CMS would be helpful to radish breeders to establish a better breeding program.

Acknowledgements This work was supported, in part, by the Crop Functional Genomics Center of the 21st Century Frontier Research Program, funded by the Ministry of Science and Technology, and by a grant of the Biogreen21 Research Program funded by the Rural Development Administration of the Republic of Korea. We also would like to thank Mr. Soo Young Chung, for his technical help with the NWB CMS line.

References

Albaum M, Luhrs R, Trautner J, Abel WO (1995) The *Tokumasu* radish mitochondrial genome contains two complete *atp9* reading frame. Plant Mol Biol 29: 179–185

Bellaoui M, Pelletier G, Budar F (1997) The steady-state level of mRNA from the Ogura cytoplasmic male sterility locus in Brassica cybrids is determined post-transcriptionally by its 3′ region. EMBO J 16(16):5057–5068

Bentolila S, Alfonso AA, Hanson MR (2002) A pentatricopeptide repeat-containing gene restores fertility to cytoplasmic male-sterile plants. Proc Natl Acad Sci USA 99:10887–10892

Bonhomme S, Budar F, Lancelin D, Small I, Defrance MC, Pelletier G (1992) Sequence and transcript analysis of the *Nco2.5* Ogura specific fragment correlated with cytoplasmic male-sterility in *Brassica* cybrids. Mol Gen Genet 235:340–348

Brown GG, Nataa F, Hua J, Richard W, Charles D, Prashant P, Martin L, Jinfa Z, Wing YC, Benoit SL (2003) The radish *Rfo* restorer gene of Ogura cytoplasmic male sterility encodes a protein with multiple pentatricopeptide repeats. Plant J 35(2):262–272

Cardi T, Earle ED (1997) Production of new CMS *Brassica oleracea* by transfer of 'Anand' cytoplasm from *B. rapa* through protoplast fusion. Theor Appl Genet 94:204–212

Grelon M, Budar F, Bonhomme S, Pelletier G (1994) Ogura cytoplasmic male-sterility (CMS)-associated *orf138* is translated into a mitochondrial membrane polypeptide in male-sterile Brassica cybrids. Mol Gen Genet 243(5):540–547

Handa H, Gualberto JM, Grienenberger JM (1995) Characterization of the mitochondrial *orfB* gene and its derivative, *orf224*, a chimeric open reading frame specific to one mitochondrial genome of the ''Polima'' male-sterile cytoplasm in rapeseed (*Brassica napus* L.). Curr Genet 28(6):546–552

Hanson MR, Bentolia S (2004) Interactions of mitochondrial and nuclear genes that affect male gemetophyte development. Plant Cell 16:S154–S169

Homme YL, Brown GG (1993) Organizational differences between cytoplasmic male sterile and male fertile *Brassica* mitochondrial genomes are confined to a single transposed locus. Nucleic Acids Res 21(8):1903–1909

Homme YL, Stahl RJ, Li X, Hameed A, Brown GG (1997) Male sterility induced by *Brassica nap* cytoplasm is correlated with expression of a chimeric gene homologous to the *pol* CMS-associated *orf224* gene. Curr Genet 31:325–335

Iwabuchi M, Koizuka N, Fujimoto H, Sakai T, Imamura J (1999) Identification and expression of the Kosena radish *(Raphanus sativus* cv. Kosena) homologue of the Ogura radish CMS-associated gene, *orf13*8. Plant Mol Biol 39:183–188

Jean M, Brown GG, Landry BS (1997) Genetic mapping of fertility restorer genes for the Polima cytoplasmic male sterility in canola (*Brassica napus* L.) using DNA markers. Theor Appl Genet 95:321–328

Kang BC, Nahm SH, Huh JH, Yoo HS, Yu JW, Lee MH, Kim BD (2001) An interspecific (*Capsicum annuum x C. chinese*) F2 linkage map in pepper using RFLP and AFLP markers. Theor Appl Genet 102(4):531–539

Koizuka N, Imai R, Fujimoto H, Hayakawa T, Kimura Y, Kohno-Murase J, Sakai T, Kawasaki S, Imamura J (2003) Genetic characterization of a pentatricopeptide repeat protein gene, *orf687*, that restores fertility in the cytoplasmic male-sterile Kosena radish. Plant J 34:407–415

Krishnasamy S, Makaroff CA (1993) Characterization of the radish mitochondrial *orfB* locus: possible relationship with male sterility in Ogura radish. Curr Genet 24:156–163

Krishnasamy S, Makaroff CA (1994) Organ-specific reduction in the abundance of a mitochondrial protein accompanies fertility restoration in cytoplasmic male-sterile radish. Plant Mol Biol 26:935–946

Makaroff CA, Palmer JD (1988) Mitochondrial DNA rearrangements and transcriptional alterations in the male-sterile cytoplasm of Ogura radish. Mol Cell Biol 8:1474–1490

Makaroff CA, Apel IJ, Palmer JD (1991) The role of *cox2*-associated repeated sequences in plant mitochondrial DNA rearrangements and radish cytoplasmic sterility. Curr Genet 19(3):183–190

Schnable PS, Wise RP (1998) The molecular basis of cytoplasmic male sterility. Trends Plant Sci 3:175–180

Singh M, Hamel N, Menassa R, Li XQ, Young B (1996) Nuclear genes associated with a single *Brassica* CMS restorer locus influence transcripts of three different mitochondrial gene regions. Genetics 143:505–516

Yamagishi H, Terachi T (1996) Molecular and biological studies on male-sterile cytoplasm in the Cruciferae. III. Distribution of Ogura-type cytoplasm among Japanese wild radishes and Asian radish cultivars. Theor Appl Genet 93:325–332

Yamagishi H, Terachi T (2001) Intra- and inter-specific variations in the mitochondrial gene *orf138* of Ogura-type male-sterile cytoplasm from *Raphanus sativus* and *Raphanus raphanistrum*. Theor Appl Genet 103:725–732