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Short Communication

Comparative studies on chloroplast DNA in Ogura-type CMS lines derived from a cybrid (*Brassica oleracea* L. var. *capitata*) and a chimera (*Brassica rapa* L. var. *peruviridis*) and on a distinction of chloroplast DNA by PCR in Brassicaceae

Gen Hattori[1], Gergely Gulyas[1], Hoytaek Kim[2], Yutaka Hirata[3,*]

[1] United Graduate School of Agricultural Science, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan; [2] Faculty of Horticulture, Chiba University, Chiba 271-8510, Japan; [3] Graduate School of Agriculture, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan
* E-mail: yhirata@cc.tuat.ac.jp Tel & Fax: +81-42-367-5625

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Abstract Two cytoplasmic male sterile (CMS) lines were found in the progenies of an intergeneric cybrid (*Brassica oleracea* L. var. *capitata*) derived from asymmetrical protoplast-fusion and an interspecific chimera (*Brassica rapa* L. var. *peruviridis*) derived from *in vitro* grafting. Although these CMS lines were different in origin, they both had abnormal anthers and aborted pollen grains. The mitochondrial DNA in these CMS lines indicated the presence of *orf138*, an indicator gene for Ogura-type cytoplasm. In this study, direct sequencing of two regions near the *rps16* in chloroplast DNA was performed. Although the sequence of the two regions in these two CMS lines was different from that in their parental lines, it was the same as the sequence in an Ogura-type CMS radish. Thus, these results suggest that the two CMS lines possess chloroplast DNA of the Ogura-type or a similar type as well as Ogura-type mitochondrial DNA. Furthermore, the region between *rps16* and *trnK* was compared among species related to *Brassica* by PCR. The result indicated the presence of many sequence variations in this region and suggested that chloroplast DNAs could be distinguished by analyzing this region by PCR.

Key words: Brassicaceae, chimera, chloroplast, cytoplasmic male sterility, protoplast-fusion.

The creation of a new variant line by protoplast-fusion, *in vitro* grafting, etc. provides a means of making genetic variability and overcoming the incompatibility of sexual crossing for plant breeding. Kameya et al. (1989) obtained a hybrid cytoplasmic male sterile (CMS) 'Ruby Ball' (*Brassica oleracea* L. var. *capitata*) from backcrossing the progenies of a cybrid line produced by asymmetric protoplast-fusion of fertile 'Ruby Ball' (*B. oleracea* L. var. *capitata* cv. Ruby Ball) with fertile radish (*Raphanus sativus* L. cv. Shogoin) (Table 1). This CMS 'Ruby Ball' was designated as a protoplast-fusion-derived CMS (PDC) line. CMS could not be restored even by crossing the line every year with fertile 'Ruby Ball'. Male sterility in plants means an inability to produce or to release functional pollen grains, as a result of a failure of formation or development of functional stamens, microspores or gametes. CMS is a maternally inherited trait, and mitochondrial rearrangement or nucleus-mitochondria incompatibility associated with CMS results in the disruption of normal pollen formation, but does not affect female fertility (Edwardson 1979; Hanson and Conde 1985). Kanno et al. (1997) reported that the RFLP pattern of the mitochondrial genome in the 'PDC' line was quite different from that of the parental lines and mentioned that DNA recombination and/or rearrangement might have occurred in many regions of the mitochondrial DNA (mtDNA). Furthermore, Motegi et al. (2003) found that the 'PDC' line possesses *orf138* (Bonhomme et al. 1992), an indicator gene for Ogura-type cytoplasm (Ogura 1968), and that the mtDNA of the 'PDC' line was very similar to an Ogura-type mtDNA by RFLP analysis using 16 known mitochondrial genes as probes. Noguchi and Hirata (1994) obtained a variant CMS line (*B. rapa* L. var. *peruviridis*) from backcrossing the progenies of mericlinal chimeras, which were induced by *in vitro* grafting of fertile 'Komatsuna' (*B. rapa* L. var. *peruviridis* cv. Komatsuna) and fertile 'Ruby Ball' (*B. oleracea* L. var. *capitata* cv. Ruby Ball) (Noguchi et al. 1992) (Table 1). This CMS 'Komatsuna' was designated as a chimera-derived CMS (CDC) line. CMS could not be restored even by crossing the line every year with fertile 'Komatsuna'. Hirata et al. (1999 and 2001) found that the 'CDC' line also possesses the Ogura-type mitochondrial genome, including *orf138*, like the 'PDC' line,

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Table 1. Plant materials in this study.

Genus	Species	Line or cultivar	Excursus	Origin or supply
Brassica	*oleracea*	'Ruby Ball'	Parental line of 'PDC' line and 'CDC' line, and maintainer of 'PDC' line	Maintained in our Lab.
	oleracea	'PDC' line	Ogura-type CMS	Maintained in our Lab. (Kameya et al. 1989; Kanno et al. 1997; Motegi et al. 2001)
	rapa	'Komatsuna'	Parental line and maintainer of 'CDC' line	Maintained in our Lab.
	rapa	'CDC' line	Ogura-type CMS	Maintained in our Lab. (Noguchi et al. 1992; Noguchi and Hirata 1994; Hirata et al. 1999 and 2001)
	amplexicaulis	1		T[1]
	barrelieri	106		T
	campestris	430		T
	carinata	103		T
	fruticulosa	103		T
	fruticulosa	401		T
	gravinae	2		T
	albograbra	O-201		T
	robertiana	101		T
	oxyrrhina	107		T
	tournefortii	165		T
	deflexa	DF-1		I[1]
	juncea	CMS line	var. *tumida*, '*nap*' type CMS	Maintained in our Lab.
	juncea		var. *tumida*, maintainer of *B. juncea* CMS line	Maintained in our Lab.
	nigra	Ni-116		I
Raphanus	*sativus*	'Shogoin'	Parental line of 'PDC' line	Maintained in our Lab.
	sativus	'MS-Gensuke'	Ogura-type CMS	Maintained in our Lab.
	raphanistrum	1		I
Diplotaxis	*catholica*	5		T
	erucoides	9		T
	harra	9		T
	siifolia	2		T
	tenuifolia	5		T
	tenuisiliqua	5		T
	virgate	4		T
Eruca	*sativa*	12		T
Erucastrum	*cardaminoides*	1		T
	abyssinicum	1		I
Moricandia	*arvensis*	10		T
Sinapis	*alba*	2		T
	arvensis	16		T

[1] These plant materials were kindly provided by Prof. Takeshi Nishio (Tohoku University *Brassica* Seed Bank) (T) and Prof. Yoshito Takahata (Iwate University) (I).

and suggested that the original 'Komatsuna' mitochondrial genome might change to the Ogura-type mitochondrial genome.

Kanno et al. (1997) reported that the RFLP pattern of the chloroplast DNA (cpDNA) in a 'PDC' line was similar to that in 'Shogoin'. However, a structural variation was detected near the region of the *rps16*, which encodes the chloroplast ribosomal protein S16, in cpDNA. This specific structure was explained to be the result of DNA recombination and/or rearrangement events (Kanno et al. 1997). However, the possibility is suggested that the 'PDC' line, and the 'CDC' line, possess the Ogura-type chloroplast genome as well as they had the mitochondrial genome of the two Ogura-type CMS lines (Ohta et al. 2006). Based on these previous data, the specific structure near the region of the *rps16* of 'PDC' line was assumed to result from the change of the chloroplast genome from the original 'Ruby Ball' type to Ogura-type.

In this study, to determine whether the specific structure of 'PDC' line is the same as that of an Ogura-type CMS radish, we attempted to clone the *rps16* region. We extracted cpDNA from 30 g of young leaves of 'PDC' line, which is the progeny of the 'PDC' line used in the previous study (Kanno et al. 1997; Motegi et al. 2003), using the method described by Triboush et al. (1998). The cpDNA of 'PDC' line was digested with *Bam*H I restriction enzyme (TaKaRa, Japan), and the cpDNA fragments were cloned into pT7 Blue vector (Novagen, USA), which was also digested with the *Bam*H I. T4

DNA Ligase (TaKaRa, Japan) was used for ligation at 16°C for 16 h, and the plasmids were transformed into *Escherichia coli* (strain DH5α). Eighteen clones were successfully isolated, one clone named clone 24 (AB288086) contained the region from *trnG* to *matK*. The *rps16* is located in this region as well. By RFLP analysis using clone 24 as a probe, the specific band of 'PDC' line was detected (data not shown). This specific band showed the same RFLP pattern as in the previous study (Kanno et al. 1997). Therefore, this variation of cpDNA in 'PDC' line is assumed to be maintained from a recent generation.

A variant region was expected in the region between *trnQ* and *rps16* or between *rps16* and *trnK* from the sequencing result of clone 24. Total genomic DNA (50 ng μl^{-1}) was extracted from 'Ruby Ball', 'Komatsuna', 'Shogoin', 'MS-Gensuke', 'PDC' line and 'CDC' line (progeny of the 'CDC' line used previously, Noguchi et al. 1992; Noguchi and Hirata 1994; Hirata et al. 1999 and 2001), by the cetyltrimethylammoniumbromide (CTAB)- based method (Aldrich and Cullis 1993), and PCR was performed with the two primer pairs (primers A and B, and C and D) to find that variation region (Table 2, Figure 1). These primers were designed based on the sequence alignments between clone 24 and the corresponding region of *Arabidopsis thaliana* (AP000423). The PCR condition was 10 min at 94°C, followed by 35 cycles of amplification consisting of 30 s at 94°C (denaturation), 30 s at 55°C (annealing), and 1 min at 72°C (extension), and 10 min at 72°C for final extension. Polymorphic fragments were detected in the region between *rps16* and *trnK* using primer C and D (Figure 2A), whereas obvious polymorphism of amplified fragments was not recognized between *trnQ* and *rps16* using primer A and B (data not shown). The amplified fragments of the two Ogura-type CMS lines were different in size from that of their parental lines but similar to that of 'MS-Gensuke'.

These amplified fragments were purified using a SUPREC-02 (TaKaRa, Japan), according to the manufacturer's instruction. Direct sequencing was performed by using an ABI Prism® 377 DNA autosequencer (Applied Biosystems, USA) with a DYEnamic ET Terminator Cycle Sequencing Kit (GE, USA). Sequence alignment was analyzed by using DDBJ CLUSTAL W (http://clustalw.ddbj.nig.ac.jp/top-j.html). As a result, the region between *rps16* and *trnK* showed variations (indels and SNPs) and many differences not only among *Brassica* species and *R. sativus* but also between 'Shogoin' and Ogura-type CMS lines. A sequence common to the Ogura-type CMS lines ('PDC' line, 'CDC' line and 'MS-Gensuke'), but different from that of 'Shogoin' was detected (No. 6, 7, 9, 13, 17 and 20 in Table 3, No. 5 in Table 4, Figure 3). In a previous study, Ohta et al. (2006) detected the sequence of 'Shogoin' type in the *matK* region from 'PDC' line and 'CDC' line, but could not obtain the sequence of 'MS-Gensuke' type, that is, Ogura-type. In this study, we detected the specific sequence of Ogura-type different from that of 'Shogoin' type in the

Table 2. Primers used in this study.

Gene region	Primer name	Primer sequence (5′–3′)
trnQ-rps16	Primer A	CTTCTATTGCCTTTGTACACC
	Primer B	GATTCGCACTAACCCTAGATCC
rps16-trnK	Primer C	GAGATCTTCGGAAGGTGGGG
	Primer D	CAATGAATGAAAACTAAGCCG
	R-type Primer	CTTTTTTAGACCTTTTTTATGACC

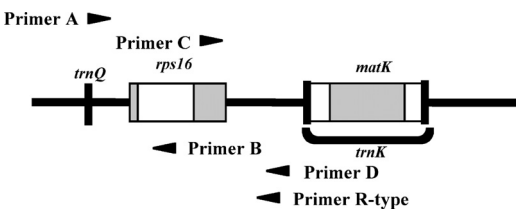

Figure 1. Schematic map of the *trnQ-trnK* locus. *Horizontal arrowheads* indicate the positions of the primers used for the amplifications of the region between *trnQ* and *rps16* (primers A and B) and the region between *rps16* and *trnK* (primers C and D).

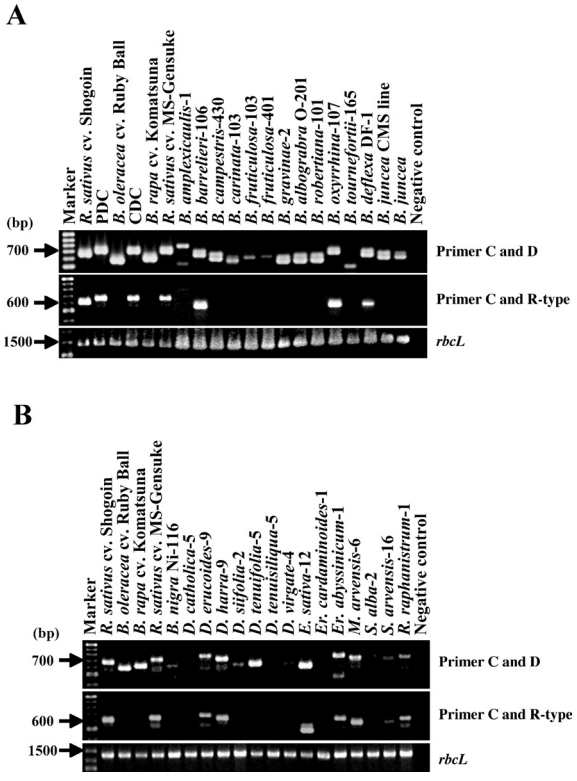

Figure 2. PCR amplifications using two primer pairs (primers C and D, and C and R-type) in Brassicaceae. Primers C and D amplified the region between *rps16* and *trnK*, and primer C and R-type primer amplified the region of radish-type between *rps16* and *trnK*. *rbcL* was used as the internal control of cpDNA. These regions were amplified in our plant materials and *Brassica* species (A) and Brassicaceae species (B). *B*: *Brassica*, *D*: *Diplotaxis*, *E*: *Eruca*, *Er*: *Erucastrum*, *M*: *Moricandia*, *S*: *Sinapis*, *R*: *Raphanus*.

Table 3. Sequence variations in the region between *trnQ* and *rps16* using primers A and B in Brassicaceae.

Accession	Length (bp)	Line or cultivar	No. 1[1]	No. 2	No. 3	No. 5	No. 6	No. 7	No. 9	No. 11	No. 12	No. 13	No. 16	No. 17	No. 19	No. 20
AB304451	691	Ruby Ball	$(C)_2$	—	A	TTAAGAAT	C	G	G	ACT	$(TA)_4$	T	TTTTTACATA	(TATAGAATTTAATGTGGG)$_1$	T	G
AB304452	693	Komatsuna	$(C)_3$	C	C	TTAAGAAT	C	G	T	AAA	$(TA)_4$	T	TTTTTACATA	(TATAGAATTTAATGTGGG)$_1$	G	G
AB304450	700	Shogoin	$(C)_2$	—	C	ATTCTT	A	G	T	TAA	$(TA)_5$	G	—	(TATACAATTTAATATGGG)$_2$	T	G
AB304449	682	MS-Gensuke	$(C)_2$	—	C	ATTCTT	C	A	G	TAA	$(TA)_5$	T	—	(TATACAATTTAATATGGG)$_1$	T	A
AB288086	682	PDC	$(C)_2$	—	C	ATTCTT	C	A	G	TAA	$(TA)_5$	T	—	(TATACAATTTAATATGGG)$_1$	T	A
	682	CDC	$(C)_2$	—	C	ATTCTT	C	A	G	TAA	$(TA)_5$	T	—	(TATACAATTTAATATGGG)$_1$	T	A

[1]There are twenty-four variations (No. 1–No. 24) in this region. The same SNPs were detected on No. 4, 8, 10, 14, 15, 18, 21, 22, 23 and 24 between 'Ruby Ball' and 'Komatsuna', and among 'Shogoin', 'MS-Gensuke', 'PDC' line and 'CDC' line.

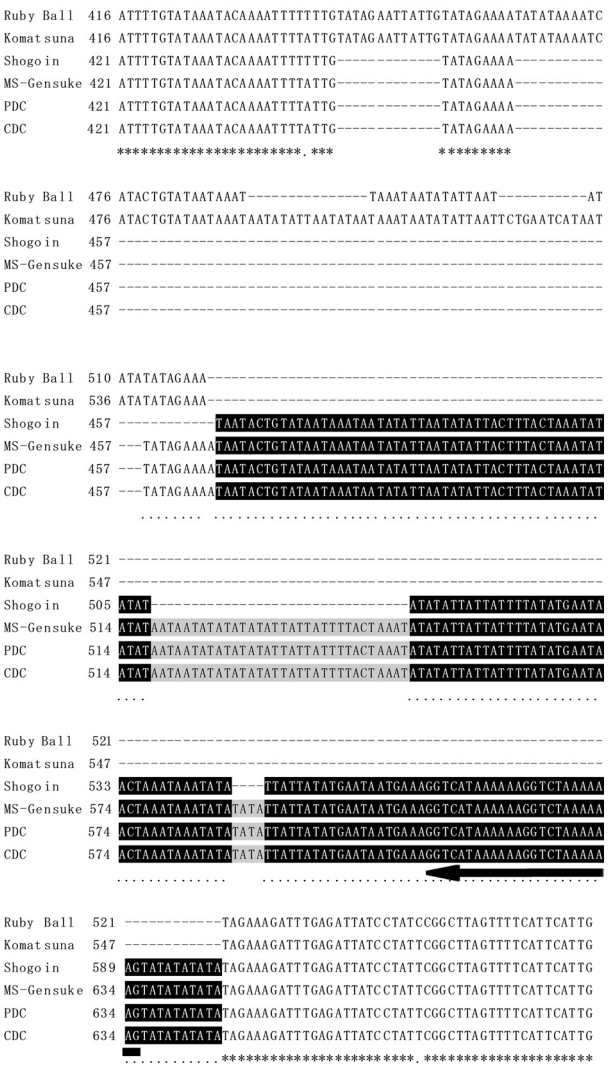

Figure 3. Sequence alignment of the partial region between *rps16* and *trnK*. *White letters* on black background indicate the radish-type specific sequence in this region. *Black letters* on gray background indicate the Ogura-type specific insertion in the radish-type specific region. *Arrowed solid line* indicates the R-type primer.

region between *trnQ* and *rps16* and between *rps16* and *trnK*, from 'PDC' line and 'CDC' line.

The radish-type specific primer (R-type primer) was designed based on the alignment of these sequences (Figure 1, 3, Table 2). Primer C and R-type primer were applied to amplify a specific fragment in the radish-type sequence. These primers amplified only the specific fragment from cpDNA in Ogura-type CMS lines and 'Shogoin' and not in 'Ruby Ball' or 'Komatsuna' (Figure 2A). These results suggested not only that different cpDNA sequences were found between two Ogura-type CMS lines ('PDC' line and 'CDC' line) and their parental lines, but also that these CMS lines possess cpDNA similar to that of 'MS-Gensuke' (Table 3, 4). Therefore, there may exist an Ogura-type chloroplast genome as well as an Ogura-type mitochondrial genome. A difference in structure, near *rps16* of cpDNA between

Table 4. Sequence variations in the amplified *rps16-trnK* region from the 1st–416th or 421st [1] nucleotide by using primers C and D in Brassicaceae.

Accession	Length (bp)	Line or cultivar	No. 2[2]	No. 3	No. 4	No. 5	No. 9
AB304455	566	Ruby Ball	T	$(TCTT)_1$	T	—	A
AB304456	592	Komatsuna	G	$(TCTT)_1$	C	—	G
AB304454	646	Shogoin	G	$(TCTT)_2$	C	G	A
AB304453	691	MS-Gensuke	G	$(TCTT)_2$	C	T	A
AB288086	691	PDC	G	$(TCTT)_2$	C	T	A
	691	CDC	G	$(TCTT)_2$	C	T	A

[1] The variations of the partial (416th or 421st–5′ terminal end) *rps16-trnK* internal region are shown in Figure 3.

[2] There are eleven variations (No. 1–No. 11) in this partial fragment. The same SNPs were detected on No. 1, 6, 7, 8, 10 and 11 between 'Ruby Ball' and 'Komatsuna', and among 'Shogoin', 'MS-Gensuke', 'PDC' line and 'CDC' line.

'PDC' line and its parental lines, was previously suggested as a result of DNA recombination and/or rearrangement (Kanno et al. 1997). Interestingly, the same sequence existed not only in the 'PDC' line and 'MS-Gensuke' but also in three Ogura-type CMS lines, including 'CDC' line, in two regions (Figure 3, Table 3, 4). The Ogura-type or similar type of cpDNAs in two Ogura-type CMS lines that were made by protoplast-fusion or *in vitro* grafting might not have been generated by DNA recombination and/or rearrangement, but by stoichiometric shifts (Small et al. 1989; Arrieta-Montiel et al. 2001), in which low-copy-number substoichiometric mtDNAs become amplified into high-copy-number dominant ones, or vice versa. There is a possibility that the Ogura-type or similar type of cpDNAs in two Ogura-type CMS lines were probably inherited from the parental line(s), which were in a heteroplasmic state having an original- and an Ogura-type (Ohta et al. 2006). However, in this study, the amplified fragment of Ogura-type specific size (~650 bp) could not be detected in their parental lines by PCR using primer C and R-type primer (Figure 2A). More detailed analyses should be performed to detect the heteroplasmic cpDNA from their parental line(s) and to understand this mechanism.

The two primer pairs detected many polymorphisms in our materials, therefore, the possibility was assumed that the region between *rps16* and *trnK* contained the variations in Brassicaceae. To examine whether the two primer pairs could be used to confirm the types of cpDNA by PCR, we analyzed the region between *rps16* and *trnK* of cpDNA from 35 lines in 30 species of 7 genera in Brassicaceae (Table 1). Some of these plant materials were kindly provided by Prof. Takeshi Nishio (Tohoku University *Brassica* Seed Bank) and Prof. Yoshito Takahata (Iwate University). In Figure 2A and 2B, using primers C and D, the amplified fragments were obtained from all *Brassica* species, but the amplified fragments from *B. fluticulosa*-103, -401 and *B. nigra* Ni-116 were more faint than that from other *Brassica* species. In species related to *Brassica*, the amplified fragments were detected from *Diplotaxis erucoides*-9, *D. harra*-9, *D. siifolia*-2, *D. tenuifolia*-5, *Eruca sativa*-12, *Erucastrum abyssinicum*-1, *Moricandia arvensis*-10, *Sinapis arvensis*-16 and *R. raphanistrum*-1. Using primer C and R-type primer, the amplified fragments were detected in *B. barrelieri*-106, *B. oxyrrhina*-107, *B. deflexa* DF-1, *D. erucoides*-9, *D. harra*-9, *E. sativa*-12, *Er. abyssinicum*-1, *M. arvensis*-10, *S. arvensis*-16 and *R. raphanistrum*-1. Although these amplified fragments (~650 bp) in *D. harra*-9, *Er. abyssinicum*-1 and *R. raphanistrum*-1 had a size similar to that in 'MS-Gensuke', the amplified fragment (~750 bp) using primers C and D in *Er. abyssinicum*-1 was different in size from that in 'MS-Gensuke' (~700 bp). The region between *rps16* and *trnK* showed polymorphism among the species in Brassicaceae.

In Brassicaceae species, many SNPs were reported in cpDNA, and these SNPs are used for classification (Yang et al. 2002; Flannery et al. 2006). These SNPs are important and valuable for taxonomy as well as evolutionary analysis of species, but a highly accurate analysis using DNA sequencing has to be performed for the detection of SNPs. However, specific or various sized amplified fragments were detected in the region between *rps16* and *trnK* by PCR using two primer pairs in many Brassicaceae species (Figure 2A, B). In this region, the type of cpDNA in a species can be confirmed using these primer pairs. It is difficult to detect the differences in cpDNA between the hybrid and its parental lines, because cpDNA sequence homology is very high, making it difficult to determine from which side of the parental lines the cpDNA in the hybrid was derived. However, the origin of the cpDNA in the hybrid may be easily determined, if the region between *rps16* and *trnK* is analyzed by PCR, using our primer pairs in Brassicaceae. Furthermore, it may be possible to apply a variation of the region near *rps16* to other plants.

The findings suggest that the organelle genomes of Ogura-type or similar type in the two CMS lines, 'PDC' and 'CDC', were derived from the parental line(s) by stoichiometric shifts. This process can be caused by stress such as tissue culture (Fauron et al. 1990) or somatic hybrid regeneration (Sakai and Imamura 1992) and is controlled by nuclear gene(s) including the *Fr* gene in *Phaseolus vulgaris* (Mackenzie and Chase 1990; Janska et al. 1998) or the *CHM* gene in *Arabidopsis* (Abdelnoor

et al. 2003). Also in our cases, nuclear gene(s) might control the copy-number of substoichiometric organelle genomes. We will perform studies to detect the substoichiometric cpDNAs of Ogura-type and/or other type from parental line(s) of two CMS lines and species related to *Brassica* based on results of the region near *rps16*, and the universality of heteroplasmic states in plants and the origin of Ogura-type organelle genome.

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