Theor Appl Genet (2013) 126:1763–1774
DOI 10.1007/s00122-013-2090-0

ORIGINAL PAPER

Complete mitochondrial genome sequence and identification of a candidate gene responsible for cytoplasmic male sterility in radish (*Raphanus sativus* L.) containing DCGMS cytoplasm

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Received: 16 December 2012 / Accepted: 19 March 2013 / Published online: 29 March 2013

Abstract A novel cytoplasmic male sterility (CMS) conferred by Dongbu cytoplasmic and genic male-sterility (DCGMS) cytoplasm and its restorer-of-fertility gene (*Rfd1*) was previously reported in radish (*Raphanus sativus* L.). Its inheritance of fertility restoration and profiles of mitochondrial DNA (mtDNA)-based molecular markers were reported to be different from those of Ogura CMS, the first reported CMS in radish. The complete mitochondrial genome sequence (239,186 bp; GenBank accession No. KC193578) of DCGMS mitotype is reported in this study. Thirty-four protein-coding genes and three ribosomal RNA genes were identified. Comparative analysis of a mitochondrial genome sequence of DCGMS and previously reported complete sequences of normal and Ogura CMS mitotypes revealed various recombined structures of seventeen syntenic sequence blocks. Short-repeat sequences were identified in almost all junctions between syntenic sequence blocks. Phylogenetic analysis of three radish mitotypes showed that DCGMS was more closely related to the normal mitotype than to the Ogura mitotype. A single 1,551-bp unique region was identified in DCGMS mtDNA sequences and a novel chimeric gene, designated *orf463*, consisting of 128-bp partial sequences of *cox1* gene and 1,261-bp unidentified sequences were found in the unique region. No other genes with a chimeric structure, a major feature of most characterized CMS-associated genes in other plant species, were found in rearranged junctions of syntenic sequence blocks. Like other known CMS-associated mitochondrial genes, the predicted gene product of *orf463* contained 12 transmembrane domains. Thus, this gene product might be integrated into the mitochondrial membrane. In total, the results indicate that *orf463* is likely to be a casual factor for CMS induction in radish containing the DCGMS cytoplasm.

Communicated by M. Xu.

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Electronic supplementary material The online version of this article (doi:10.1007/s00122-013-2090-0) contains supplementary material, which is available to authorized users.

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Introduction

Plant mitochondrial genomes have several peculiar features, such as an extended genome size, frequent rearrangement, and an extremely low rate of point mutations when compared with animal and fungal mitochondrial genomes (Budar et al. 2003; Knoop 2004; Kubo and Newton 2008). The genome size of plant mitochondrial DNA (mtDNA) is generally larger and more variable than

that of animal mtDNA. In contrast to compact animal mitochondrial genomes, which generally range in size from 15 to 18 kb, the sizes of most compact plant mitochondrial genomes are much larger, such as 208 kb in dicot *Brassica hirta* (Palmer and Herbon 1987) and 228 kb in monocot *Spirodela polyrhiza* (Wang et al. 2012). Furthermore, an extremely large-sized 11,318,806 bp mitochondrial genome was recently reported in *Silene conica* (Sloan et al. 2012). Apart from their variable size, the precise configuration of plant mitochondrial genomes is still elusive. Multiple linear or mixture of linear and circular forms have been described (Backert et al. 1997; Oldenburg and Bendich 2001; Allen et al. 2007).

Among the peculiar features of plant mitochondrial genomes, dynamic rearrangement of genome is the most conspicuous character. Repeat sequences are known to mediate recombination causing rearrangement. Relatively, large repeats exceeding 1 kb are involved in frequent formation of multipartite structure from a putative master chromosome. On the other hand, short repeats smaller than 1 kb drive the dynamic rearrangement of plant mitochondrial genomes (Palmer 1988; Small et al. 1989; Albert et al. 1998; Woloszynska and Trojanowski 2009). Short repeat-mediated recombination produces a variety of sub-genomic molecules, which are driving forces for the evolution of plant mitochondrial genomes (Small et al. 1989; Kmiec et al. 2006). For example, transition from the *cis*- to *trans*-splicing of group II intron of mitochondrial genes results from mtDNA rearrangement (Qiu and Palmer 2004; Bonen 2008; Kim and Yoon 2010).

The relative copy number or stoichiometry of subgenomes varies in a single plant cell and specific stoichiometry of subgenomes is maintained throughout generations (Sakai and Imamura 1993; Bellaoui et al. 1998; Janska et al. 1998; Arrieta-Montiel et al. 2001; Kim et al. 2007). Nuclear genes, such as *Msh1* (Abdelnoor et al. 2006), *RecA* (Shedge et al. 2007), and *OSB1* (Zaegel et al. 2006) control subgenome transmission and maintain its specific stoichiometry. Sandhu et al. (2007) showed that the inactivation of the *Msh1* gene via RNA interference induces a change in the stoichiometry of subgenomes in tobacco and tomato. Arrieta-Montiel et al. (2009) also reported extensive reorganization of mitochondrial genomes in two *Msh1* mutants of *Arabidopsis*. The change in stoichiometry of subgenomic mtDNA is also known to be induced by tissue culture (Kanazawa et al. 1994).

Short repeat-mediated mtDNA rearrangement often produces aberrant chimeric genes consisting of partial sequences of known mitochondrial genes and unidentified sequences. If these chimeric genes acquire a proper promoter or they are co-transcribed with genuine mitochondrial genes, these chimeric genes can be expressed and aberrant protein products might affect the normal function of mitochondria. A typical phenotype expressed by these chimeric genes is cytoplasmic male-sterility (CMS), a maternally inherited inability to produce viable pollen grain in plants (Schnable and Wise 1998; Budar et al. 2003; Hanson and Bentolila 2004). Naturally occurring CMS has been reported in more than 140 plant species and have been used in production of F_1 hybrid seed in many crop species (Laser and Lersten 1972).

Male sterility conferred by aberrant mitochondrial genes can be reversed to be male-fertile by the action of nuclear gene(s), which are often designated restorer-of-fertility (Rf) genes. Several nuclear *Rf* genes that have been cloned encode pentatricopeptide repeat (PPR) protein (Bentolila et al. 2002; Komori et al. 2004; Klein et al. 2005), but maize *Rf2* (Cui et al. 1996), rice *Rf17* (Fujii and Toriyama 2009), and *Rf2* (Itabashi et al. 2011) encode aldehyde dehydrogenase, a protein with unknown function, and a glycine-rich protein, respectively, suggesting involvement of complex interaction between mitochondrial CMS-inducing genes and nuclear *Rf* genes for restoration of male fertility.

In radish (*Raphanus sativus* L.), a CMS system reported by Ogura (1968) has been most extensively characterized. The mitochondrial gene, *orf138*, responsible for Ogura CMS was identified by investigating the correlation between male-fertility phenotypes of cybrid-derived progenies and specific mtDNA regions (Bonhomme et al. 1991, 1992; Grelon et al. 1994). Nuclear *Rf* gene, *Rfo*, was also isolated by map-based cloning approaches and was revealed to code for a PPR protein (Brown et al. 2003; Desloire et al. 2003; Koizuka et al. 2003). In addition, the complete mitochondrial genome sequences of Ogura and normal mitotypes were recently reported (Tanaka et al. 2012).

A new CMS system designated as Dongbu cytoplasmic and genic male sterility (DCGMS) was recently reported (Lee et al. 2008). Male-sterility phenotype and the abnormal pollen development stage in the DCGMS mitotype were different from those of Ogura CMS. Profiles of molecular markers developed for radish mitotype classification also showed that DCGMS was different from Ogura CMS (Kim et al. 2009; Lee et al. 2009). Fertility restoration of male sterility conferred by the DCGMS mitotype is controlled by a single nuclear locus, *Rfd1*, and this *Rf* locus is not linked to the *Rfo*, the restorer gene for Ogura CMS (Kim et al. 2010; Cho et al. 2012).

In this study, we obtained a complete mitochondrial genome sequence of the DCGMS mitotypes and a candidate mitochondrial gene responsible for male-sterility induction in DCGMS was identified through comparative genomics analysis of complete mitochondrial genome sequences of three radish mitotypes.

Materials and methods

Plant materials and mitochondrial DNA extraction

Radish breeding lines containing DCGMS and Ogura cytoplasm whose mitotypes were previously confirmed by molecular markers developed for radish mitotype classification (Lee et al. 2008; Kim et al. 2009) were used for bacterial artificial chromosome (BAC) library construction. Seeds were germinated in the dark condition and etiolated whole plants were harvested for mitochondrial DNA extraction.

Mitochondrial DNA was extracted using the method described by Millar et al. (2001) with some modifications. Harvested plants were ground in a mortar and homogenized with 500 ml of grinding buffer (0.3 M mannitol, 50 mM Tris–HCl, 3 mM EDTA, 8 mM cysteine, 1 mM 2-mercaptoethanol, 0.1 % bovine serum albumin, 1 % PVP-40) on ice. Homogenized cells were filtered through one layer of Miracloth and four layers of cheesecloth, and centrifuged twice at 15,000*g*. After centrifugation, the pellet was loaded on to 28 % Percoll gradient buffer and centrifuged at 40,000*g* for 80 min. The obtained pale green layer was harvested and washed with wash buffer comprised 0.3 M mannitol and 50 mM Tris–HCl. Isolated mitochondria were treated with proteinase and mtDNA was obtained.

BAC library construction and mitochondrial genome sequencing

Prepared intact mtDNA was partially digested with *Sau*3AI enzyme for 30 min at 37 °C. Digested DNA was separated through clamped homogeneous electrical field (CHEF) electrophoresis twice and fragments of approximately 50–100 kb were selected and purified DNA was ligated to a pCUGIBAC–BamHI cloning vector (Luo et al. 2001). The ligated vector was transformed into DH10B cells. Clones containing mitochondrial genomes were screened with known mitochondrial genes (*cox1*, *atp4*, *atp6*, *atp8*, and *nad3*) as probes. The positive clones were further confirmed by BAC end sequencing.

DNA pools of selected BAC clones were sequenced using the GS-FLX system (Roche Applied Science, Indianapolis, IN, USA) and sequencing reads were assembled using Newbler Assembler Software Version 2.3 (454 Life Sciences, Branford, CT, USA). A final complete circle sequence assembly was confirmed by PCR and sequencing for 12 regions. The position of selected BAC clones and sequencing depth are shown in Supplementary Fig. 1. The known mitochondrial genes were identified using a BLAST search and tRNAs were identified using tRNAscan-SE Version 1.21 (Schattner et al. 2005). The putative open reading frames (ORFs) with a minimum size of 70 codons were found using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html).

Construction of a phylogenetic tree and prediction of transmembrane domains

Conserved sequence blocks of approximately 40-kb conserved sequence blocks of three radish mitotypes and four *Brassica* species were aligned using BioEdit software (Hall 1999). The gaps were removed using Gblocks software (Castresana 2000) with the options for less stringent selections. The phylogenetic trees were constructed using *MEGA* Version 4 (Tamura et al. 2007) with the maximum parsimony method. Node support for the phylogenetic tree was assessed using 1,000 bootstrap replicates. The transmembrane domains of the putative CMS-inducing gene were predicted using the TMHMM server version 2.0 (Krogh et al. 2001).

Polymerase chain reaction (PCR) and sequencing of PCR products

PCR was performed in 10-μL reaction mixtures containing 0.05 μg template, 1 μL 10× PCR buffer, 0.2 μL forward primer (10 μM), 0.2 μL reverse primer (10 μM), 0.2 μL dNTPs (10 mM each), and 0.1 μL polymerase mix (Advantage 2 Polymerase Mix, Clontech, Palo Alto, CA, USA). The primers used in this study are listed in Supplementary Table 1. PCR amplification was performed with an initial denaturation at 95 °C for 4 min, ten cycles of 95 °C for 30 s, 67 °C (0.8 °C decrements in each cycle) for 30 s, and 72 °C for 2 min, 30 cycles of 95 °C for 30 s, 59 °C for 30 s, and 72 °C for 2 min, and a final 7-min extension at 72 °C. PCR products were visualized on 1.5 % agarose gels after ethidium bromide staining. PCR products were purified using a QIAquick PCR Purification kit (QIAGEN, Valencia, CA, USA) and sequenced directly. Sequencing reactions were carried out using big dye (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol and the sequences were obtained using an ABI PRISM 3730XL Analyzer (Applied Biosystems).

Rapid amplification of cDNA ends (RACE)

Total RNA was extracted from two-leaf stage seedlings containing DCGMS mitotype using an RNeasy Plant Mini Kit (QIAGEN) following the manufacturer's instructions. RACE was carried out with a commercial RACE kit (SMART RACE cDNA Amplification Kit; Clontech) according to the manufacturer's instructions. The purified RACE PCR products were either sequenced directly or

after cloning into a TOPO TA cloning vector for sequencing (Invitrogen, Carlsbad, CA, USA).

Results

Complete mitochondrial genome sequence of DCGMS cytoplasm in radish

A BAC library was constructed using mtDNA isolated from radish containing DCGMS cytoplasm. Among 1,152 BAC clones, 38 clones containing mitochondrial sequences were selected by hybridization using the known mitochondrial genes *cox1*, *atp4*, *atp6*, *atp8*, and *nad3* as probes. Sequences of the selected BAC clones were finally assembled as a 239,186-bp circular form (Fig. 1). The DCGMS mitochondrial genome sequence was deposited to GenBank under accession number KC193578. The DCGMS mitochondrial genomes contained 33 protein coding genes, three ribosomal RNA genes, and one additional *atp9* gene. In addition, 38 ORFs coding for hypothetical proteins were identified (Supplementary Table 2). The known mitochondrial gene content of the DCGMS mitotype is identical to that of the Ogura mitotype, but the normal mitotype is reported to contain one entire copy and partial sequences of *atp9* gene (Tanaka et al. 2012). There was one-point mutation causing non-synonymous conservative amino acid change between two *atp9* genes in the DCGMS mitotype, in contrast to the Ogura mitotype, in which the sequences of both *atp9* genes were identical.

Comparative analysis of three radish mitochondrial genomes

A total of 17 syntenic sequence blocks were identified by comparison of three complete mitochondrial genome sequences of normal (AB694743), Ogura (AB694744), and DCGMS mitotypes (Fig. 2). The position and orientation of sequence blocks were highly rearranged among the three mitotypes. The 9,731-bp large repeat identified in the Ogura mitotype was also identified, but the orientations of these large repeats were inverted in the DCGMS mitotype. The DCGMS mitotype lacked the 5,536-bp sequence block 16 and 1,538-bp block 17, which were present in both the normal and Ogura mitotypes. The DCGMS mitotype contained the smallest mitochondrial genome (239,186-bp) compared with those of the normal (244,036-bp) and Ogura (258,426-bp) mitotypes. The size difference between DCGMS and the other two mitotypes mostly resulted from the lack of sequence block 16.

In addition to mtDNA sequences of the DCGMS mitotype, a complete mtDNA sequence of Ogura mitotype was also obtained based on the same approach for mitochondrial genome sequencing of DCGMS mitotype in this study. An independent BAC library was constructed for mitochondrial genome of Ogura mitochondrial genome and sequences of pooled BAC clones were assembled as one circular sequence. The gene organization of the Ogura mtDNA sequence was identical to that reported by Tanaka et al. (2012) except for a 79,976-bp inversion (Fig. 2). At the end of this inversion, a 311-bp repeat sequence was identified with an inverted orientation. To verify the correct organization of the Ogura mtDNA sequences, PCR amplifications were carried out using primers binding to two sequence blocks flanking each boundary of this inverted region. PCR amplification showed that both organizations were present in Ogura mtDNA together at almost an equimolar level (Fig. 3b). These results imply that there might be two isomeric master circles in Ogura mitochondrial genomes. The two isomeric master circles can be produced by recombination between two putative small subgenomic circles as depicted in Fig. 3a. Merging of SC1 and SC2 with two possible orientations might produce two isomeric mater circles: MC1 and MC2 (Fig. 3a). To assess the possibility of existence of these two putative small circles, PCR amplifications were carried out using primers binding to two sequence blocks flanking the recombination breakpoints of SC1 and SC2. PCR results showed that both subgenomes existed in Ogura mtDNA, but the copy number of both forms was very low since they were observed as faint bands (Fig. 3b). Sequences of these PCR products confirmed identities of these organizations.

Because it is known that dynamic mtDNA rearrangement is caused by recombination mediated by short repeats less than 1 kb (Small et al. 1989; Kmiec et al. 2006), short repeats were searched for around the junctions between syntenic sequence blocks. Short repeats ranging from 79 to 521 bp in size were identified in most junctions (Table 1). Two junctions, including the sequence block 5 might be rearranged via the 9,731-bp large repeats. A 16-bp short repeat was found at the end of sequence block 2 in the junction between block 2 and the 1,550-bp unique region. No repeat sequence was found only in the junction between blocks 1 and 2, but a 110-bp sequence of this junction was repeated in the Ogura mitochondrial genome.

To assess phylogenetic relationship among three radish mitotypes, the 40-kb syntenic sequence block 2, the largest sequence block of three mitotypes, were used for the construction of phylogenetic tree. Because block 2 sequences were also found to be conserved in *Brassica* species, approximately 40-kb corresponding homologous sequences from *B. napus*, *B. oleracea*, *B. rapa*, and *B. juncea* were included in tree construction as outgroups. The phylogenetic tree showed that the DCGMS mitotype was more closely related to the normal mitotype than to the Ogura mitotype (Fig. 4). Comparison of the number of

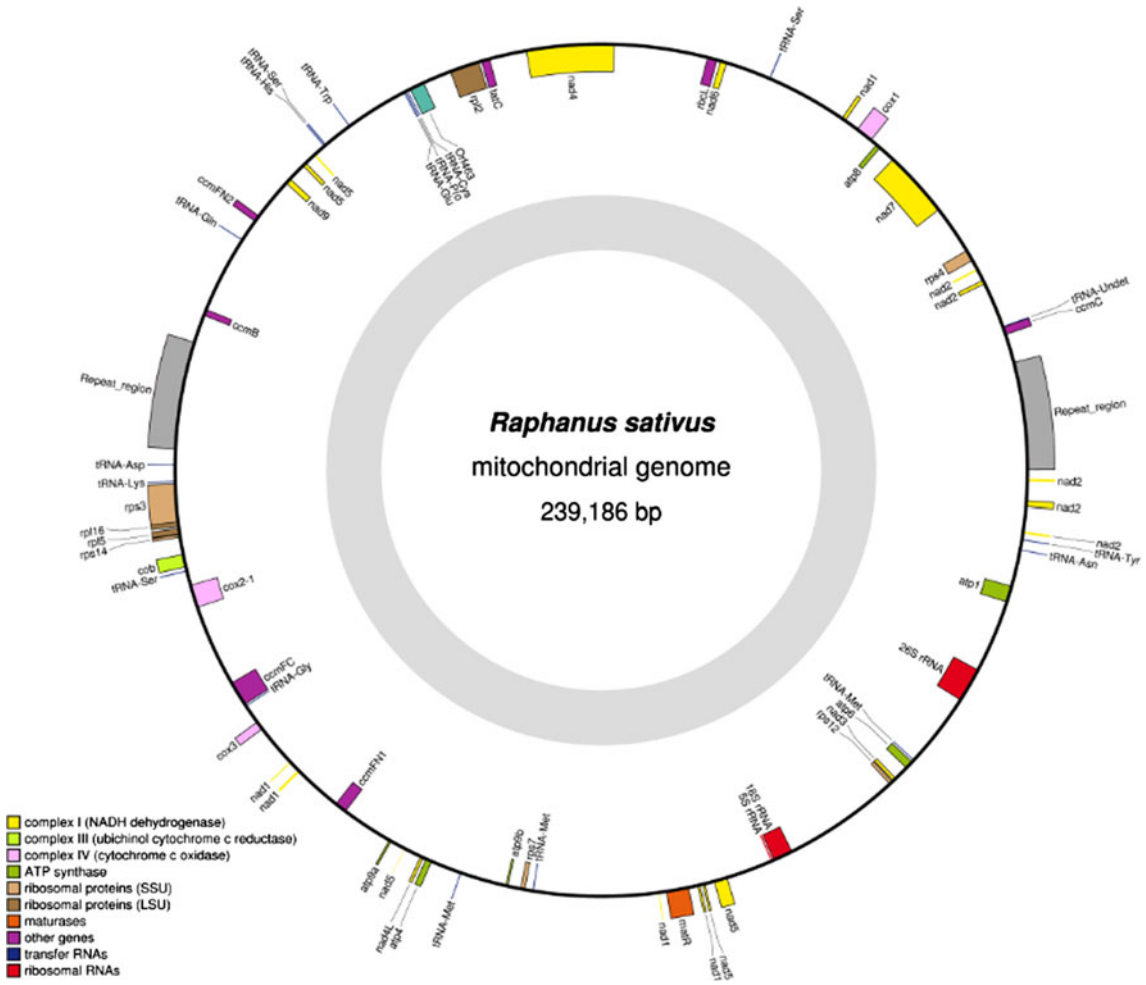

Fig. 1 Gene organization of the complete mitochondrial genome of radish containing DCGMS mitotype. Genes with forward and reverse orientation are shown in outside and inside of the *circle*, respectively. The exact positions of each gene are shown in Supplementary Table 2

nucleotide substitutions in whole mitochondrial genomes also showed close relationship of the DCGMS and normal mitotypes (Table 2). In particular, sequences of *atp6* gene of both the DCGMS and normal mitotypes were identical, but the 28-bp 5′ end of *atp6* in the Ogura mitotype was highly variable due to the rearrangement occurring at the 5′ end of the *atp6* gene.

Identification of a candidate gene responsible for male-sterility induction in the DCGMS mitotype

Because the mtDNA sequence of the DCGMS mitotype was more similar to that of normal mitotype, comparison of the two mtDNA sequences was performed to identify unique sequences. A 1,551-bp sequence between syntenic sequence blocks 2 and 3 and a 451-bp sequence between blocks 5 and 6 were unique to the DCGMS mitotype when compared with the normal mitotype. However, the 451-bp sequence was identified in the Ogura mitotype with 100 % nucleotide sequence identity. As to the 1,551-bp region, no homologous sequence was identified in Ogura mitotype and even in the public GenBank database. Only sequence smaller than 70-bp showed approximately 80 % homology with whole-genome shotgun sequences of *Vitis vinifera*.

A novel 1,389-bp ORF was identified in the 1,551-bp unique sequence and at the end of sequence block 3. Hereafter, we designated this novel gene as *orf463*. The 128-bp 5′ sequences, including the start codon was present in the sequence block 3, but most ORF sequences resided in the unique region (Fig. 5a). Interestingly, the 128-bp 5′ sequence of this novel ORF was identical to the 5′ end of the *cox1* gene. In addition, a 119-bp sequence containing putative 5′-untranslated region (UTR) was identical to that of *cox1* gene except for a single point mutation. Beyond this putative 5′UTR sequence, dissimilar sequence blocks 3 and 1 were connected to *orf463* and *cox1*, respectively (Fig. 5a).

To investigate the expression of *orf463* and to identify transcription start and stop sites, RACE PCR was performed. The sequence of the 5′ RACE product of *orf463*

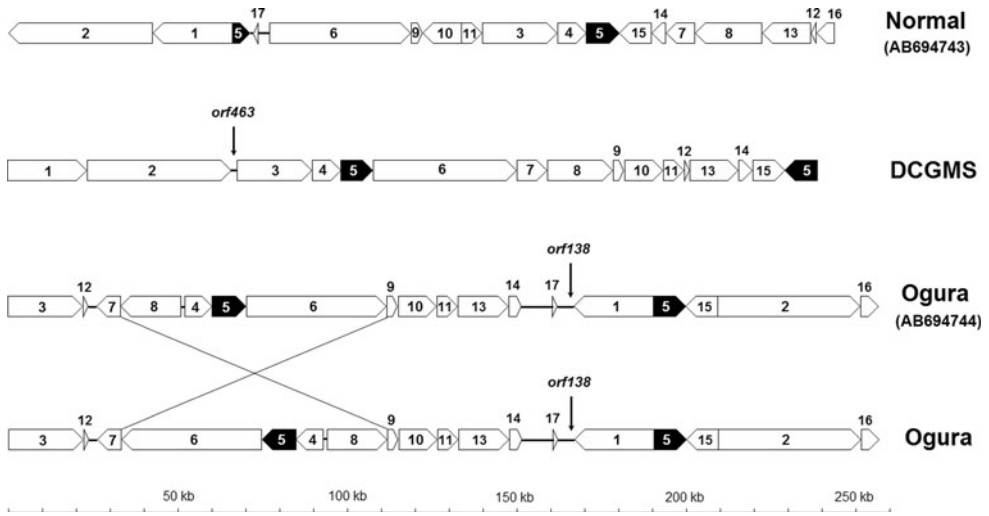

Fig. 2 Organization of syntenic sequence blocks of three radish mitochondrial genomes. The position and orientation of each sequence block are shown to scale in *arrow-shaped boxes*. The *filled boxes* represent two large repeats. The unique sequences are shown as *thick lines*. The positions of *orf138* and *orf463* are indicated with *vertical arrows*. The ends of a large inverted region between two Ogura sequences are connected with *vertical lines*. The normal and Ogura mtDNA sequences reported by Tanaka et al. (2012) are shown with *GenBank accession numbers* in *parenthesis*

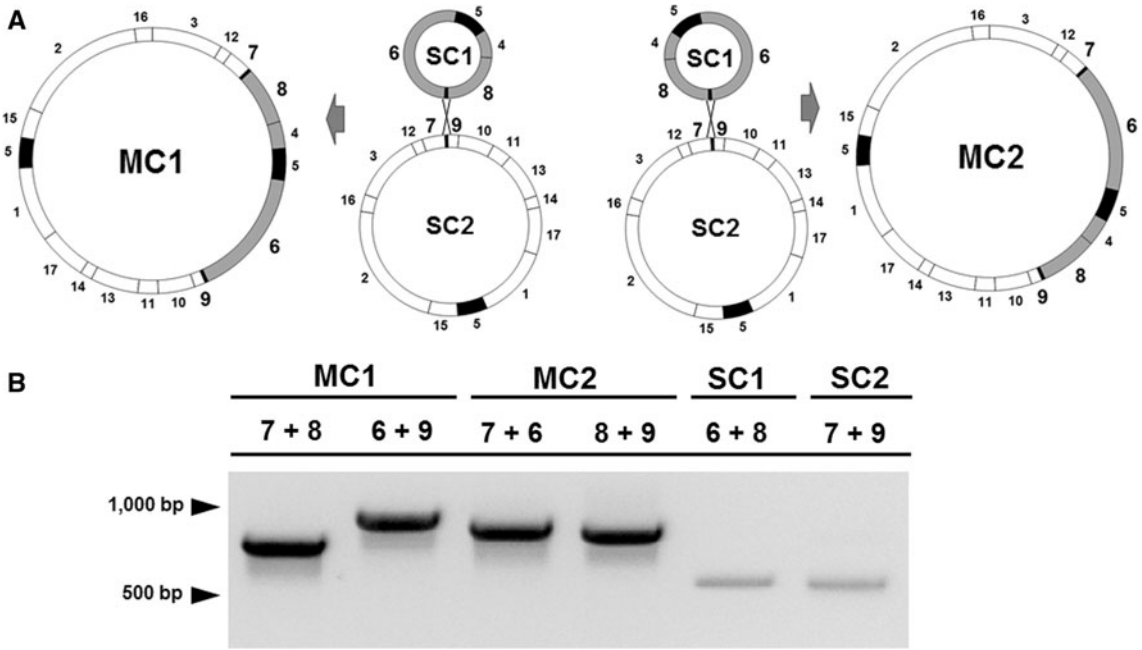

Fig. 3 Multipartite structure of the Ogura mitochondrial genome. **a** Schematic diagrams showing *two master circles* and *two subgenomes*. *Circles* are drawn to scale. MC and SC imply *master* and *subgenomic circles*, respectively. *Numbers outside circles* indicate the syntenic sequence blocks shown in Fig. 2. *Vertical lines* between SC1 and SC2 show the position of recombination. The *filled boxes* indicate repeat sequences. The sequence blocks consisting of SC1 are shown as *gray boxes* in MC1 and MC2. **b** PCR products amplified using primers biding to sequence blocks flanking the inversion. 7 + 8, 6 + 9, 7 + 6, 8 + 9, 6 + 8, and 7 + 9 represent combinations of primers binding to corresponding sequence blocks shown in Fig. 3a

showed that transcription started at 219-bp upstream of the start codon (Fig. 5b). Because no known gene was co-transcribed with the *orf463*, it was assumed that the *orf463* might acquire its own promoter. As to 3′ RACE, two positive RACE products were observed (Fig. 5b), but the smaller one did not include the stop codon. Therefore, the larger transcript might serve as a primary transcript for translation of the *orf463*. Like most CMS-associated genes reported in other species, the predicted protein product of *orf463* contained 12 transmembrane domains (Fig. 6),

Table 1 Short repeat sequences identified on the junctions between syntenic sequence blocks

Junctions	Repeat		
	Name	Length	Frequency in DCGMS mtDNA
B1–B2	R1	145	3
Unique sequence–B3	R1	145	3
B4-B5	R2	232	3
B5–B6	Large repeat	9,731	2
B6–B7	R3	311	2
B7–B8	R4	87	2
B8–B9	R5	83	3
B9–B10	R6	147	2
B10–B11	R6	147	2
B11–B12	R1	145	3
B12–B13	R7	141	2
B13–B14	R4	87	2
B14–B15	R8	521	2
B15–B5	Large repeat	9,731	2
B5–B1	R9	79	3

B1–B15: syntenic sequence blocks shown in Fig. 2

implying that the protein product might be integrated into mitochondrial membrane.

Identification of sequence variations on known and putative coding genes between DCGMS and normal mitochondrial sequences

In addition to 34 known protein-coding genes, 38 ORFs coding for hypothetical protein containing at least 70 residues were identified in the DCGMS mitochondrial genome (Supplementary Table 2). Sequence variations in these 72 genes between the DCGMS and normal mitotypes were searched for to identify any critical mutations implicated in male-sterility induction in the DCGMS mitotype. One or two single nucleotide polymorphisms were identified in seven known mitochondrial genes (Table 3). Point mutations in *rpl2* and *rpl16* were synonymous, causing no amino acid change. Sequences of *nad4* and *ccmFC* were identical to those of the Ogura mitotype, although there were one or two point mutations between the DCGMS and normal mitotypes (Table 3). The point mutations on *nad7*, *rps3*, and *matR* resulted in amino acid changes, but the changed amino acids might not be positioned on the conserved regions because the changed amino acids were also found in several other plant species (data not shown). Therefore, point mutations on these seven known genes may not be related with male-sterility induction in the DCGMS mitotype.

Among 38 ORFs encoding hypothetical proteins, seven ORFs were found to contain sequence variations between the normal and DCGMS mitotypes. The sequence of HP (hypothetical protein) 53 was identical to that of the Ogura mitotype. Although there were 1-4-bp point mutations in six ORFs between the DCGMS and normal mitotypes, no chimeric ORF created by mtDNA rearrangement was identified in the DCGMS mitotype, except for *orf463*. Most known CMS-associated genes contain chimeric ORFs (Hanson and Bentolila 2004). In addition, homologous sequences of six ORFs were also found in mitochondrial genomic regions that were not related with CMS in *Brassica* species and *Arabidopsis* (data not shown). In total, these results showed that there were no significant sequence variations on the protein-coding genes between the normal and DCGMS mitotypes except for the *orf463*. Therefore, it is assumed that *orf463* might be responsible for male-sterility induction in the DCGMS mitotype. However, we cannot rule out the possibility that mutations on six ORFs might be involved in CMS induction. Further functional studies of *orf463* and six ORFs are required to elucidate the exact mechanism of CMS induction in DCGMS mitotype.

Table 2 The number of nucleotide substitutions present on the normal and Ogura mtDNAs compared with that of DCGMS mitotype

Mitotype	Nucleotide substitutions		
	Coding region[a]	Non-coding region	Total
Ogura	10	184	194
Normal	8	83	91

[a] Nucleotide substitutions present on the Ogura *ap6* gene were excluded due to highly variable 5′ end sequences

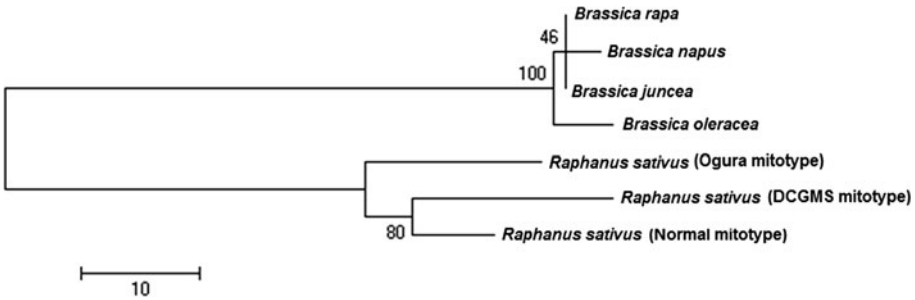

Fig. 4 Phylogenetic tree of three radish mitotypes and four *Brassica* species constructed using 40-kb block 2 sequences. The numbers at the nodes are the bootstrap probability (%) with 1,000 replicates. The *scale bars* indicate nucleotide substitutions per site

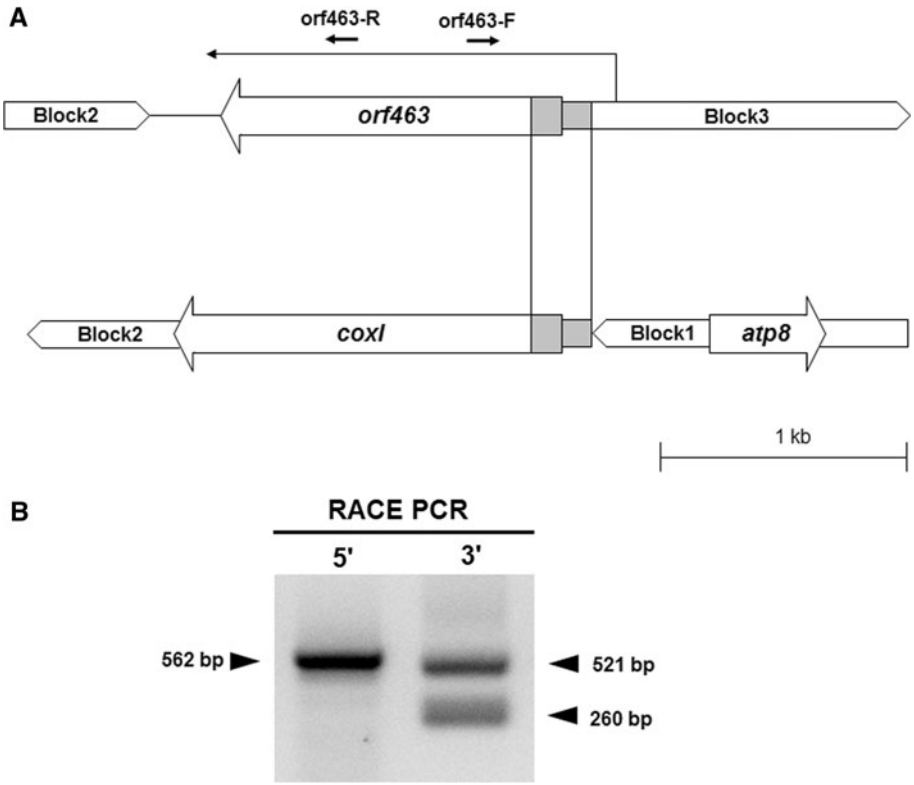

Fig. 5 Schematic representation of *orf463* gene structure and its flanking regions. **a** Organization of *orf463* and *cox1* genes in DCGMS mitotypes. Homologous sequences between the *orf463* and *cox1* genes are shown in *gray boxes*. *Arrow-shaped boxes* indicate the 5′- to 3′-direction. *Horizontal short arrows* indicate PCR primer-binding sites for RACE. A *long horizontal arrow* shows positions of transcription start and stop sites. **b** 5′ and 3′ RACE PCR products amplified using universal primer mix and two primers shown in Fig. 5a

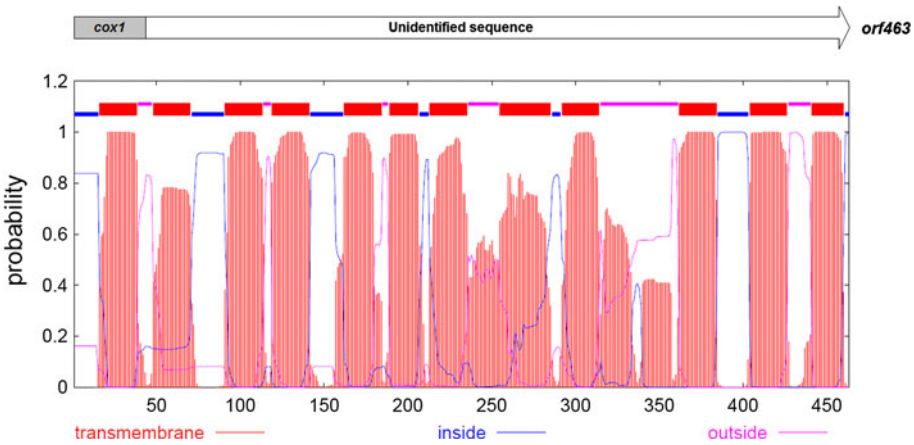

Fig. 6 Locations and probability of transmembrane domains of the *orf463* gene product. The output of the TMHMM server is shown underneath the *orf463* diagram

Discussion

Comparative analysis of complete mitochondrial genome sequences of three radish mitotypes

With the help of advanced sequencing technologies and assembly software, the number of complete mitochondrial genome sequences deposited in the GenBank database has increased to 69 (Wang et al. 2012) since the first plant mitochondrial genome sequence was reported in *Arabidopsis* (Unseld et al. 1997). Complete mitochondrial genome sequences of radish DCGMS and Ogura mitotypes were produced by construction of BAC libraries in this study. Meanwhile, complete mtDNA sequences of normal and Ogura mitotypes were recently produced by shotgun sequencing (Tanaka et al. 2012). The normal mitotype used by Tanaka et al. (2012) might be identical to the DBRMF2 mitotypes reported in the previous studies (Lee et al. 2008; Kim et al. 2009). The cytoplasm types of 120 radish germplasms introduced from diverse countries were classified into four mitotypes (Ogura, DCGMS, DBRMF1, and DBRMF2) using molecular markers previously developed on the basis of mtDNA and cpDNA polymorphisms (Lee et al. 2008; Kim et al. 2009). All normal male-fertile radish accessions contain either DBRMF1 or DBRMF2 mitotypes, and phylogenetic analysis of four mitotypes showed

Table 3 Sequence variations of mitochondrial genes coding for known and hypothetical proteins (HPs) between normal and DCGMS mitotypes

Gene	Position from the start codon	Nucleotide variation		Amino acid change
		Normal	DCGMS	
nad7	3,831	G	A	V → I
nad4	1,010	T	C	L → P
rpl2	204	G	A	Synonymous
rps3	551	G	A	G → E
rpl16	492	G	A	Synonymous
ccmFC	605	AG	GT	E → G
matR	1,547	G	A	T → I
HP 01	231	–	C insertion	Frame shift
HP 16	265	G	A	G → R
	546	C	T	Synonymous
	861	G	C	Synonymous
HP 21	279	CA	2-bp deletion	Frame shift
	339	C	T	Synonymous
HP 22	285	A	T	Stop → W
HP 52	187	A	C	I → L
	192	GA	TC	KI → NL
	202	G	C	V → L
	225	C	A	Synonymous
HP 53	185	–	G insertion	Frame shift
HP 71	119	–	CT insertion	Frame shift

that Ogura and DCGMS mitotypes were close to DBRMF1 and DBRMF2, respectively (Kim et al. 2009). Therefore, the normal mtDNA sequence reported by Tanaka et al. (2012) might correspond to the DBRMF2 mitotype because phylogenetic analysis of normal, Ogura, and DCGMS mitotypes showed that the DCGMS mitotype was closer to the normal mitotype than Ogura mitotype in this study. Furthermore, *cox1–atp8* linkage and a 910-bp region containing a short-repeat cluster in the 3′ flanking sequence of *atp6* which were present in DBRMF2, but not in DBRMF1 (Kim et al. 2007) were found in the normal mtDNA sequence reported by Tanaka et al. (2012). If the complete mtDNA sequences of the DBRMF1 mitotype were obtained in the near future, comparative analysis of four radish mitotypes would elucidate their phylogenetic relationship and dynamic mtDNA rearrangements among four radish mitotypes.

The mtDNA organization of Ogura mitotypes reported by Tanaka et al. (2012) was identical to that of Ogura sequences produced in this study except for an 80-kb inversion. We verified that both organizations were present at similar level in Ogura mtDNA. This implies that putative master circles presented by Tanaka et al. (2012) and this study might exist as multipartite forms. Multipartite structures of mtDNA have been commonly observed in other plant species. As an example, the tobacco mitochondrial genome consists of multipartite organization with two isomeric master circles and six subgenomic circles (Sugiyama et al. 2005). In addition, the comparative analysis of complete mtDNA sequences of normal and WA-CMS mitotypes in rice also showed that many rearrangements specific to one mtDNA were also detected in the other mitotype genome at the substoichiometric level (Bentolila and Stefanov 2012). Similarly, although we identified two isomeric master circles and two substoichiometric subgenomes, there might be more diverse subgenomes in Ogura and other radish mitotypes because numerous possible rearrangements among 17 syntenic sequence blocks could occur through repeat sequence-mediated recombination.

Identification of a candidate gene responsible for induction of male-sterility in the DCGMS mitotype

Through comparative analysis of three complete radish mitochondrial genome sequences, we identified a chimeric gene, *orf435*, which might be responsible for male-sterility induction in the DCGMS mitotype. Many CMS-inducing mitochondrial genes have been identified in plant species and four strategies have been utilized to prove their association with CMS induction (Hanson and Bentolila 2004). The analysis of somatic hybrids generated from protoplast fusion between CMS and fertile lines was used to identify mtDNA regions that co-segregated with fertility phenotypes. The *orf138* gene causing CMS in the Ogura mitotype was identified through analysis of segregating somatic hybrid progenies produced from protoplast fusion between Ogura CMS radish and *B. napus* (Bonhomme et al. 1991, 1992). The second uncommon strategy is comparing proteomes of CMS and fertile lines. The *urf13* gene responsible for male sterility in CMS-T cytoplasm of maize was initially detected by this strategy (Forde et al. 1978).

The most common strategy is searching for different mitochondrial transcript profiles or gene organizations between CMS and fertile lines, and examining the effect of nuclear *Rf* genes on the function of candidate CMS-inducing genes at the transcriptional or post-transcriptional level. Many CMS-associated mitochondrial genes have been identified using this strategy (Hanson and Bentolila 2004). In fact, we initially identified *orf463* by searching for different mtDNA organizations between the normal and DCGMS mitotypes linked to the short repeat sequences (data not shown). The short repeat sequences were previously identified during development of molecular markers for distinction of radish mitotypes (Lee et al. 2009) and mtDNA regions linked to these repeats were obtained by genome walking.

The last strategy is the comparative genomics approach, in which CMS and fertile genomes are as compared to identify the mtDNA region unique to the CMS mitotype. This approach has been not so successful because both CMS and normal genomes are so divergent that multiple mitotype-specific mtDNA regions were found (Hanson and Bentolila 2004). However, if CMS and normal mitochondrial genomes were close enough to show just a few differences, CMS-associated genes could be detected by this strategy. As one example, the CMS-inducing gene was identified in common bean by comparing CMS and normal lines created by recent reversion from the same CMS progenitor (Mackenzie and Chase 1990). As a second example, the mtDNA region associated with pol CMS in *B. napus* was also identified by comparing closely related normal and CMS mtDNA organizations (L'Homme and Brown 1993). *orf463*, a putative CMS-associated gene in radish DCGMS mitotype was identified by comparative genomics strategy in this study. We present several results supporting the involvement of *orf463* in male-sterility induction. First, only two unique mtDNA regions were found in DCGMS mitochondrial genome when compared with that of the normal mitotype, but a 451-bp region between sequence blocks 4 and 5 were also found in the Ogura mtDNA region, which was not related with CMS. Therefore, we could delimit the unique region within a 1,551-bp interval and the *orf463* was found in that unique sequence. Similarly, Allen et al. (2007) identified two already known CMS-associated genes for CMS-S and CMS-T in the unique regions specific for each CMS mitotype by comparative analysis of complete mitochondrial genome sequences of two fertile and three CMS maize lines.

Second, no other chimeric ORFs were found in rearranged junctions of syntenic sequence blocks, except for *orf463*, although there were several rearrangements between normal and DCGMS mtDNAs. Except for a few cases, such as radish *orf138*, almost all CMS-inducing mitochondrial genes reported so far have been chimeric genes consisting of partial sequences of known mitochondrial genes and unidentified sequences (Hanson and Bentolila 2004; Chase 2007). With regard to six ORFs showing polymorphisms between DCGMS and normal mitotypes, they contained only 1 to 4-bp nucleotide changes and homologous sequences were also found in *Brassica* species and *Arabidopsis*. In most cases, CMS-associated ORFs contained unidentified sequences (Hanson and Bentolila 2004; Chase 2007). Allen et al. (2007) also identified 95–116 ORFs with unknown function, but none were associated with CMS. In addition, it was reported that most mitochondrial ORFs with unknown function were not stably transcribed in *Arabidopsis* (Giegé et al. 1998) and maize (Meyer 2004). For these reasons, these six ORFs may not be related with CMS.

Finally, 12 predicted transmembrane domains were identified in the gene product of *orf463*. Because transmembrane domains were evenly distributed throughout the entire protein, this protein might be integrated into the mitochondrial membrane. Likewise, most CMS-associated mitochondrial genes have been shown to contain transmembrane domains (Hanson and Bentolila 2004). In total, the *orf463* is likely to be involved in CMS induction in DCGMS mitotype. However, transcription of *orf463* seemed not to be affected by the presence of the dominant nuclear *Rf* gene (data not shown). Therefore, the study of the interaction between *orf463* and nuclear *Rf* gene at the post-transcriptional level must be performed to elucidate a precise role of *orf463* in CMS induction.

Acknowledgments This work was supported by a Grant from the Next-Generation BioGreen 21 Program (Plant Molecular Breeding Center No. PJ007992), Rural Development Administration and a grant (108169-05-01-SB010) from the Technology Development Program for Agriculture and Forestry, Ministry of Agriculture and Forestry, Republic of Korea.

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