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ORIGINAL PAPER

Identification of mitochondrial genome rearrangements unique to novel cytoplasmic male sterility in radish (*Raphanus sativus* L.)

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Abstract A novel cytoplasmic male-sterility (CMS) radish (*Raphanus sativus* L.) and its associated mitotype (DCGMS) were previously identified; however, no mtDNA fragments flanking the *atp6* gene were found in the DCGMS mitotype. Unlike three other mitotypes in this study, a unique mtDNA organization, *atp6–nad3–rps12*, was found to be the major mtDNA structure associated with this mitotype. This organization may have arisen from short repeat sequence-mediated recombination events. The short repeat clusters involved in the mtDNA rearrangement around the *atp6* gene also exist as repetitive sequences in the complete mitochondrial genomes of other members of the Brassicaceae family, including rapeseed and Arabidopsis. These sequences do not exist as repetitive elements in the mtDNA of tobacco, sugar beet, or rice. While studying the regions flanking *atp6*, we identified a truncated *atp6* mtDNA fragment which consists of the 5′ part of the *atp6* gene linked to an unidentified sequence. This mtDNA structure was present in all mitotypes; however, a single nucleotide insertion mutation leading to a frame-shift was identified only in the DCGMS mitotype. Although this truncated *atp6* organization was transcribed, there was no significantly different expression between male-sterile and fertile segregating individuals from the BC_1F_1 population originating from a cross between male-sterile and restorer parents. Comprehensive survey of the single base-pair insertion showed that it was maternally inherited and unique to the DCGMS mitotype. Therefore, this single nucleotide polymorphism (SNP) in the coding sequence of the mtDNA will be a useful molecular marker for the detection of the DCGMS mitotype.

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Introduction

A number of characteristics unique to plant mitochondrial genomes have been revealed in higher plants (for reviews, see Fauron et al. 1990; Budar et al. 2003; Hanson and Bentolila 2004; Knoop 2004). In contrast to animal mitochondrial genomes whose sizes are 15–18 kb, plant mitochondrial genomes are relatively large and of variable sizes. For example, *Brassica hirta* was reported to have a mitochondrial genome size of 208 kb (Palmer and Herbon 1987), whereas muskmelons retain 2,400 kb of mtDNA (Ward et al. 1981). Aside from genome size, the complexity of plant mitochondrial genomes is also unusual. Indeed, the exact configurations of plant mitochondrial genomes are still unclear. Few plant mitochondrial genomes appear to be a single circular form, but instead they tend to consist of multigenomic linear and circular molecules or head-to-tail concatemer structures (Oldenburg and Bendich 1998, 2001).

In spite of their complex organizations, the complete sequences of the master circles of higher plant mitochondrial genomes have been obtained from *Arabidopsis* (Unseld et al. 1997), sugar beet (Kubo et al. 2000), rice

(Notsu et al. 2002), rapeseed (Handa 2003), and tobacco (Sugiyama et al. 2005). These sequences revealed a frequent loss and acquisition of mtDNA from nuclear or plastid genomes and a ubiquitous presence of repeat sequences. For example, in rice mtDNA, 26% of the total genome consists of repeat sequences of lengths ranging from 3.1 to 127.6 kb (Notsu et al. 2002). Homologous recombination between the large repeat sequences helps to drive the creation of multipartite structures consisting of varied subgenomic molecules (Palmer 1988; Albert et al. 1998); therefore, these repeat sequences have likely been a driving force in plant mitochondrial genome evolution.

The stoichiometry of subgenomic molecules is also different depending on mitotypes (mitochondrial genome variant) within the same species (Sakai and Imamura 1993; Bellaoui et al. 1998; Kim et al. 2007). Some substoichiometric mtDNA molecules are present at a level of less than one copy per 100 cells (Arrieta-Montiel et al. 2001). The specific stoichiometry of subgenomic molecules seems to be constant among mitotypes and throughout generations (Janska et al. 1998; Kim et al. 2007). Unidentified genetic factors are thought to be involved in maintenance of the stoichiometry. However, the stoichiometry sometimes changes through genomic shifting (Small et al. 1989; Arrieta-Montiel et al. 2001). Although the mechanisms of genomic shifting are still unclear, several factors including tissue culture (Fauron et al. 1990) and nuclear genes (Mackenzie and Chase 1990; Janska et al. 1998; Abdelnoor et al. 2003) have been known to trigger it.

Meanwhile, short repeat sequences (>100 bp in length) distributed throughout the entire mitochondrial genome are responsible for dynamic mtDNA rearrangements that result in the creation of a variety of substoichiometric molecules (Albert et al. 1998; Kim et al. 2007). Small et al. (1989) proposed the importance of substoichiometric intermediates in the rapid evolution of plant mitochondrial genomes. These short repeat-mediated mtDNA rearrangements have been involved in the creation of novel chimeric genes. In fact, most male-sterility inducing genes in higher plants are chimeric genes (Hanson 1991; Hanson and Bentolila 2004).

Cytoplasmic male-sterility (CMS) plants are unable to produce viable pollen grains, and thus they have been exploited in F_1 hybrid seed production in many economically important crops. In radishes, since Ogura (1968) first reported CMS, a number of F_1 hybrid cultivars have been developed using the Ogura CMS system. Furthermore, Ogura CMS has been introduced into *Brassica* species through protoplast fusion to establish a stable F_1 hybrid breeding system (Pelletier et al. 1983; Menczel et al. 1987; Jarl et al. 1989). The mitochondrial chimeric gene responsible for Ogura CMS, *orf138*, as well as its restorer-of-fertility gene, *Rfo*, has been isolated (Bonhomme et al. 1991; Grelon et al. 1994; Brown et al. 2003; Desloire et al. 2003; Koizuka et al. 2003). The *Rfo* gene of radish has been introduced into *B. napus* to produce fertile F_1 rapeseed cultivars containing the *orf138* gene (Pellan-Delourme and Renard 1988; Sakai et al. 1996; Primard-Brisset et al. 2005).

Despite extensive studies on Ogura CMS, few efforts to search for new sources of CMS have been made in radishes. However, recently, a new CMS and its restorers-of-fertility were reported (Lee et al. 2008). This novel CMS contains a new mitotype different from previously reported radish mitotypes (Kim et al. 2007). The present study reports the identification and characterization of the gene organization flanking the *atp6* gene in this new mitotype. Additionally, our data indicate the involvement of short repeat sequences in dynamic mtDNA rearrangements.

Materials and methods

Plant materials

New radish CMS lines reported in a previous study (Lee et al. 2008) were used as plant materials containing the DCGMS mitotype. The F_1 hybrids between DCGMS lines and male parents possessing DBRMF1 mitotypes, and their BC_1F_1 populations were used to verify maternal inheritance of the SNP marker unique to the DCGMS mitotype. The genomic DNAs of six breeding lines used in the previous study (Kim et al. 2007) were used as representatives of three mitotypes. The BC_1F_1 populations [DCGMS × (DCGMS × restorer lines)] in which male-sterile and fertile individuals segregated were used to estimate the effect of restorer-of-fertility gene(s) on the expression of the newly identified mtDNA transcripts. Thirteen DCGMS-derived lines and 24 radish breeding lines containing other mitotypes were used in the survey of the SNP marker on the truncated *atp6* gene.

DNA extraction and PCR amplification

Total genomic DNA was extracted from the leaf tissue of three-leaf stage radishes using a commercial DNA extraction kit (DNeasy Plant Mini Kit, QIAGEN, Valencia, CA, USA) according to the manufacturer's manual. PCR was performed in a 10-μL reaction mixture containing 0.05 μg template, 1.0 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). PCR amplification was carried out with an initial denaturation step at 94°C for 5 min followed by 40 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 90 s, and a final 10-min extension at 72°C. The primer sequences used in this study are presented in Table 1.

Table 1 Primer sequences used in this study

Primer names	Primer sequences (5′–3′)
PF1	AAGTGGGTTCGCTTGGACTATGCTATG
PR1	GCCTGTTCCCTCTGAATCGGTTGATTG
PF2	AATGTGGTTTCGATCCTTCCGGTGATG
PR2	CTGCACCATATTTGGATCTGCCGCTTC
PR3	CGGCTTGAATGTGGGCTAATTGGATG
PF4	ATACCTCGGGGAAGAAGCGGGGT
PR4	TAGCCATTTGGTGTGACCTCTGACCG

Sequencing of the PCR products and SNP marker genotyping

Following PCR amplification, the PCR products were purified using the QIAquick PCR Purification kit (QIAGEN). The purified PCR products were either sequenced directly or following cloning into the pGEM T-easy cloning vector (Promega, Madison, WI, USA). 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 3700 Genetic Analyzer (Applied Biosystems). The SNP marker was genotyped by direct sequencing after purification of PCR products.

Genome walking for isolation of sequences flanking the *atp6* gene

To isolate flanking sequences of the *atp6* gene, genome walking was performed using the Universal GenomeWalker Kit (Clontech) according to the manufacturer's protocol. Genome walking libraries were constructed from the genomic DNA of male-sterile lines containing the DCGMS mitotypes.

Reverse transcription-PCR and rapid amplification of cDNA ends

Total RNA was extracted from unopened flowers of five male-sterile and five fertile BC_1F_1 segregating individuals using the TRI® Reagent RNA isolation kit (Sigma, St Louis, MO, USA) following the manufacturer's instructions. cDNA was synthesized from total RNA using a commercial cDNA synthesis kit (SuperScript™ first-strand synthesis system for RT-PCR, Invitrogen, Carlsbad, CA, USA). RT-PCR amplification was carried out with an initial denaturation step at 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 2 min, and a final 10-min extension at 72°C. RACE was carried out with a commercial RACE kit (SMART RACE cDNA Amplification Kit; Clontech) according to the manufacturer's instructions.

Results

Identification of a new mtDNA organization linked to the *atp6* gene in DCGMS radishes

In previous studies (Kim et al. 2007; Lee et al. 2008), we reported a new radish CMS, mitotype, and set of molecular markers used for the classification of the four mitotypes (Ogura, DBRMF1, DBRMF2, and DCGMS). Interestingly, molecular markers amplifying the *atp6*-flanking regions of the three other mitotypes failed to amplify any clear PCR products in the DCGMS mitotype (Fig. 1). This result led us to speculate that there might be a rearrangement of the mtDNA around the *atp6* gene in the DCGMS mitotype. To isolate the region flanking *atp6*, we performed genome walking and found that one major PCR product could be obtained from the 3′ genome walk. Unlike in the other three mitotypes, the *atp6* gene was found to be linked to the *nad3* and *rps12* genes (Fig. 2a). PCR amplification confirmed that the newly identified *atp6–nad3–rps12* gene organization is the major mtDNA structure harboring the *atp6* gene in the DCGMS mitotype (Fig. 1). However, the *atp6–nad3–rps12* organization also appeared to be present in other mitotypes, although the intensities of the PCR products were consistently lower than that observed in the DCGMS mitotype.

The newly identified *atp6* flanking organization was likely created by a short repeat sequence-mediated recombination event. We hypothesize that the repeat (R3) at which the crossing-over might have occurred is the same as the one involved in a double crossing-over event which was reported to generate the major structural distinction between the DBRMF1 and DBRMF2 mitotypes (Fig. 2b) (Kim et al. 2007). In summary, a single recombination between the R3 repeat of DBRMF2 and the *nad3–rps12*

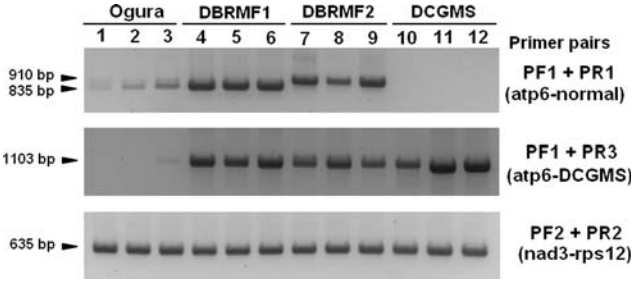

Fig. 1 PCR amplification products of mtDNA fragments from four radish mitotypes. The positions of each primer are depicted in Fig. 2a. *1–3*, *4–6*, *7–9*, *10–12* representative cultivars or breeding lines for each mitotype

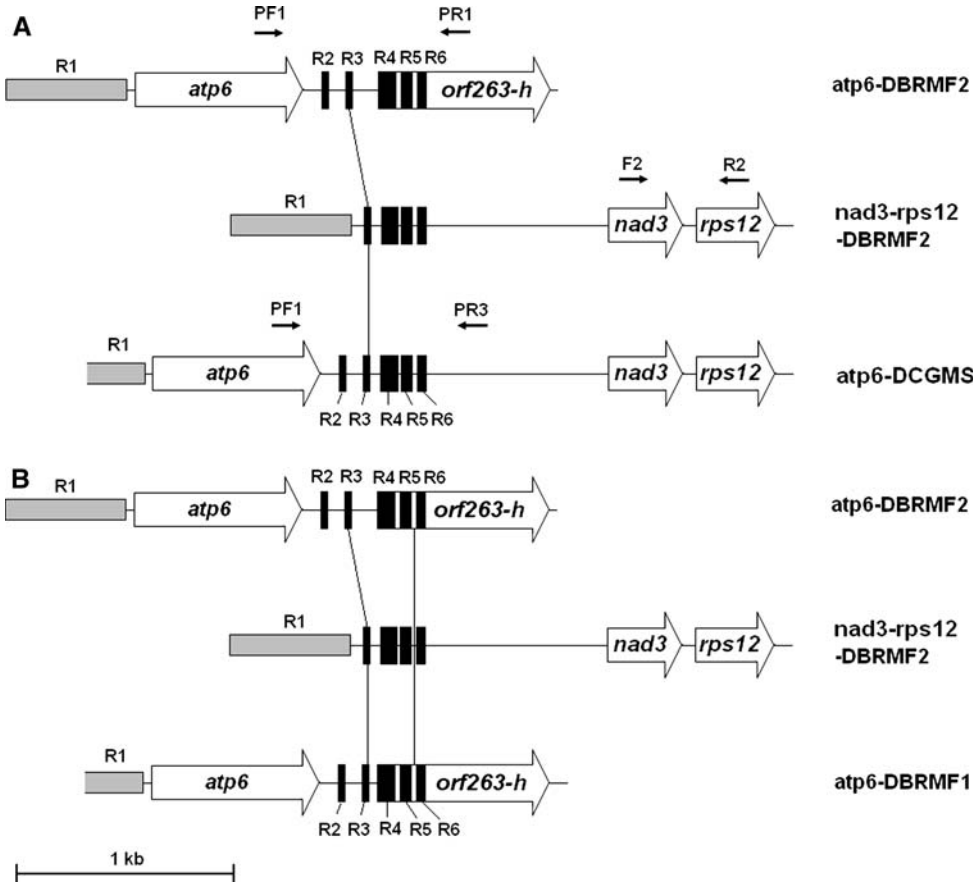

Fig. 2 The organization of mitochondrial genome regions linked to the *atp6* gene in three different mitotypes. **a** Comparison of *atp6*-flanking sequences between the DBRMF2 and DCGMS mitotypes. *orf263-h*: homologous sequence of *Brassica tournefortii orf263* gene. Arrow-shaped boxes indicate the 5′–3′ direction. The *rectangular, gray boxes* (*R1*) indicate repeat sequences. The *filled rectangular boxes* (*R2–R6*) are short repeat sequences. *Horizontal arrows* indicate primer binding sites. **b** Sequence comparison of the DBRMF1 and DBRMF2 mitotypes. This figure was adapted from Kim et al. (2007)

regions appears to have resulted in the *atp6–nad3–rps12* gene organization of the DCGMS mitotype. In contrast, a double recombination in the same region appears to have generated the repeat cluster (R3–R6) length difference between DBRMF1 and DBRMF2 mitotypes.

Presence of the radish short repeats in mitochondrial genomes of other plant species

Previously, we showed that the radish short repeat cluster (R2–R6) was directly involved in dynamic mitochondrial genome rearrangements around the *atp6* gene. We next determined if these repeats could also be found in other radish mitochondrial genome sequences. When we examined the mtDNA sequences of *Raphanus sativus*, we noted the presence of portions of this repeat cluster in multiple regions. For example, the flanking region of *coxI* contains the repeat region from R2 to R3 (Fig. 3a, b), and the repeat R2–R3–R4 cluster was also found in the 3′ flanking region of the *atp9* gene (GenBank accession X69319).

The short repeat cluster (R2–R6) was also found in the flanking sequence of the *atp6* gene of *Brassica tournefortii* (Fig. 3a, b). The repeats are located in the *orf263* gene which has been reported to be a male-sterility inducing gene in alloplasmic male-sterile *Brassica* lines (Landgren et al. 1996). The start codon of the *orf263* is positioned inside the R3 short repeat. The same ‘ATG’ sequence in the R3 repeat is also a start codon of the radish *coxI* gene (Fig. 3b). Furthermore, a chimeric organization which consists of the 5′ part of radish *coxI* gene and the 3′ part of *B. tournefortii orf263* gene was identified in 3′ flanking sequence of the *atp6* gene in the DBRMF2 mitotype (Fig. 3a). The putative breakpoints of the cross-over events are all positioned within the short repeats (Fig. 3a). In addition, the *orf263* homolog whose start codon is also located in the R3 repeat was identified in the *orf286* on the mitochondrial genome of *B. napus* (GenBank accession AP006444). The presence of short repeats in multiple regions implies that they may be ubiquitously present in radish mitochondrial genomes, and that they may play a key role in dynamic mtDNA rearrangements. However, the exact frequency of short repeats will not be known until the complete sequencing of the radish mitochondrial genome is accomplished.

To obtain an estimate of the frequency of these clusters, we searched for the short repeats in the mitochondrial genomes of five other plant species whose complete mitochondrial genomes have been sequenced (Table 2). The short repeat, R2, appeared 14 times throughout mitochondrial genome of *Brassica napus*, which is a close

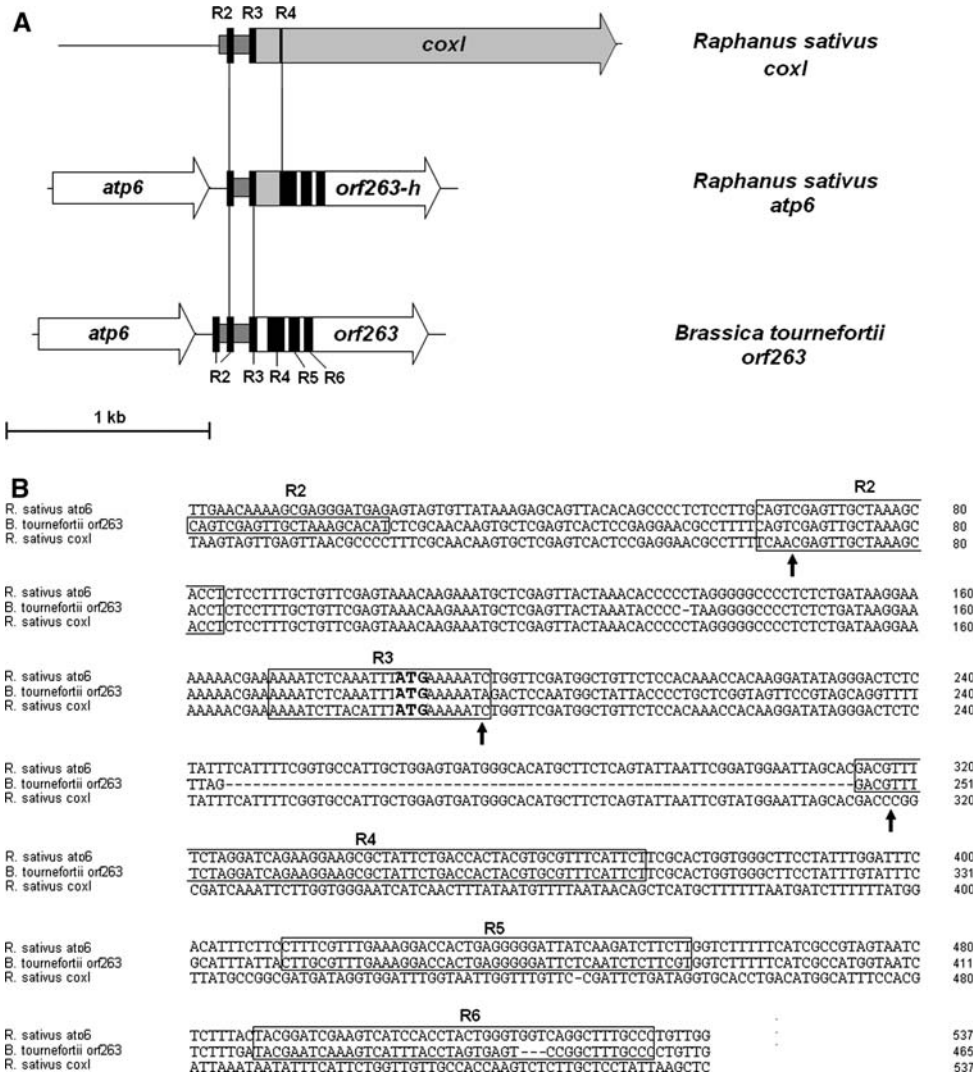

Fig. 3 The organization of mitochondrial genome regions indicates a role of short repeat sequences in mtDNA rearrangement. **a** Comparison of three mtDNA units harboring common short repeats. *Arrow-shaped boxes* indicate the 5′–3′ direction. The *filled rectangular boxes* (*R2–R6*) are short repeat sequences. *orf263-h*: homologous sequence of *Brassica tournefortii orf263* gene. **b** Nucleotide sequence alignment of three mtDNA regions containing short repeat sequences. The short repeat sequences are enclosed with *rectangular boxes*. The *vertical arrows* indicate the putative breakpoints in crossing-over events

Table 2 Frequency of radish short repeat sequences in the complete mitochondrial genomes of other plant species

Plant species	Frequency of homologous repeat sequences				
	R2 (22 bp)[a]	R3 (24 bp)	R4 (57 bp)	R5 (45 bp)	R6 (44 bp)
Brassica napus	14 (95–100)[b]	2 (95–100)	3 (98–100)	2 (91)	2 (92–97)
Arabidopsis thaliana	6 (86–100)	3 (91–95)	5 (91–96)	3 (75–77)	5 (88–92)
Nicotiana tabacum	0	1 (83)	1 (89)	0	0
Beta vulgaris	0	0	1 (91)	0	0
Oryza sativa	0	0	2 (90)	0	0

[a] Length of repeat in nucleotides

[b] Ranges of nucleotide sequence identity with radish repeats. The GenBank accession numbers of complete mitochondrial genomes of other species are AP006444 (*Brassica napus*), Y08501 (*Arabidopsis thaliana*), BA000042 (*Nicotiana tabacum*), BA000024 (*Beta vulgaris*), BA000029 (*Oryza sativa*). The homologous repeats were identified using BLAST 2 SEQUNECES (Tatusova and Madden 1999)

relative of radish. Additionally, the mtDNA of Arabidopsis, which belongs to the same Brassicaceae family as radish, contained between three and six instances of the short repeats. However, in distantly related plant species such as tobacco, sugar beet, and rice, rather than re-iterated instances of these sequences, there were either single homologous sequences or no homologous regions at all (Table 2).

Identification of a novel truncated *atp6* gene organization

When the 3′ genome walking was performed to obtain the flanking sequence 3′ of the *atp6* gene in the DCGMS mitotype, an additional fragment distinct from the *atp6–nad3–rps12* unit was isolated. This fragment contained only the 5′ part of the *atp6* gene, indicating that the open reading frame of the *atp6* gene was truncated at 255 bp from the start codon (Fig. 4a, b). The 3′ sequences linked to the truncated *atp6* gene are not similar to known mtDNA sequences. In fact, BLAST searches identified as the best match a 285-bp sequence block from the nuclear genome of Arabidopsis (GenBank accession AC010155) that is 73% identical to the sequence in question.

Unlike the other mtDNA rearrangements mediated by short repeat sequences presented in this study, no repeat sequences were identified around the breakpoint of the *atp6* truncation. PCR amplification of the truncated *atp6* fragment from four radish mitotypes showed that they all contain this fragment, although the copy number appeared to vary between mitotypes (Fig. 4c).

Meanwhile, RT-PCR experiments showed that this fragment is transcribed, though the expression level is very low (data not shown). In order to exclude the possibility that the RT-PCR results are an artifact resulting from mtDNA contamination, the transcription of this fragment was confirmed by 5′ RACE PCR amplification of its 5′ end. The predicted translation of the putative transcript derived from the novel 225 bp ORF reveals that a protein product smaller than the normal ATP6 protein would be produced (Fig. 4b). Hereafter, we designate this putative ORF as *orf225*.

Like other male-sterility related mitochondrial genes, most of which are in close proximity to the *atp6* and *atp9* genes (Hanson and Bentolila 2004), this novel *orf225* gene might be related to the male sterility of the DCGMS mitotype. To examine the effect of nuclear restorer-of-fertility gene(s) on the expression of the *orf225*, the expression level was compared between male-fertile and sterile segregating BC_1F_1 individuals originating from a cross between a male-sterile DCGMS female line and a fertile male parental line containing restorer-of-fertility gene(s) for DCGMS male sterility. However, no significantly different expression

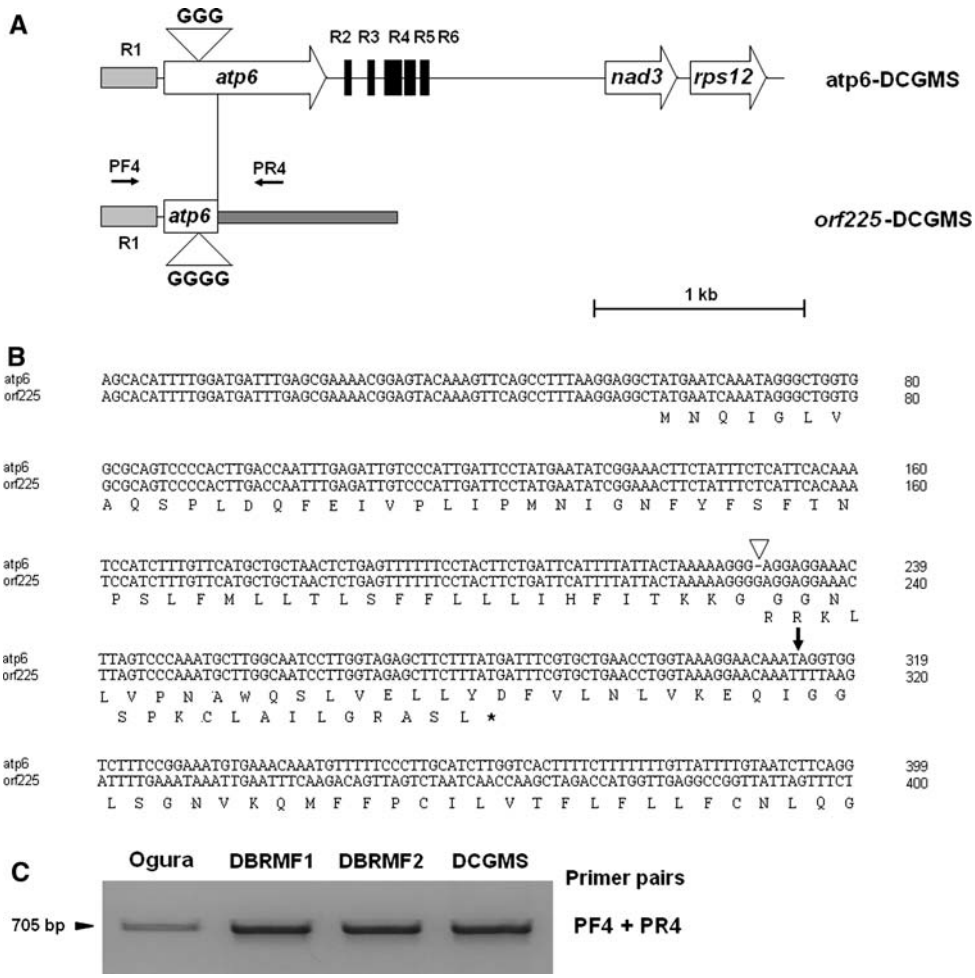

Fig. 4 Sequence comparison of the normal and the truncated *atp6* genes. **a** Comparison of mtDNA structures between the normal and truncated forms of *atp6*. *Arrow-shaped boxes* indicate the 5′–3′ direction. The *rectangular, gray boxes* (*R1*) on the 5′ end of the *atp6* gene indicate repeat sequences. The *rectangular box* linked to the truncated *atp6* indicates the unknown sequence. The *filled rectangular boxes* (*R2–R6*) are short repeat sequences. *Horizontal arrows* on the sequences indicate the primer binding sites. **b** Nucleotide and deduced amino acid sequence alignment of the normal and truncated *atp6* genes. The *triangle* indicates the position of the insertion mutation. The *vertical arrow* indicates the position of breakpoint of the putative cross-over. Only different amino acid sequences between the *orf225* and the normal *atp6* gene were presented. **c** PCR amplification products of mtDNA fragments from four radish mitotypes. The position of each primer is depicted in Fig. 4a

was observed between fertile and sterile plants (data not shown).

Identification of a SNP marker unique to DCGMS male-sterile radishes

The 5′ sequence of *orf225* (572 bp) obtained from RACE was identical to that of the normal *atp6* gene except for a single nucleotide insertion in the DCGMS mitotype (Fig. 4a, b). This insertion was not found in any of the other three mitotypes (Fig. 5). The 5′ sequences of the other mitotypes were completely identical to those of the normal *atp6* gene. The inheritance of the insertion was surveyed from the original DCGMS lines to BC_1F_1 populations using direct sequencing of the fragment containing *orf225*. The results showed that all individuals containing DCGMS cytoplasm possessed the single nucleotide insertion, proving the stable inheritance of the SNP. The F_1 hybrid between a female parent harboring the DCGMS mitotype and a male parent whose mitotype was DBRMF1 was always positive for the insertion (Fig. 5), indicating the presence of the *orf225* sequence in cytoplasmic genomes. Therefore, the single nucleotide insertion which is unique to the DCGMS mitotype will be a useful molecular marker for the specific detection of the DCGMS mitotype.

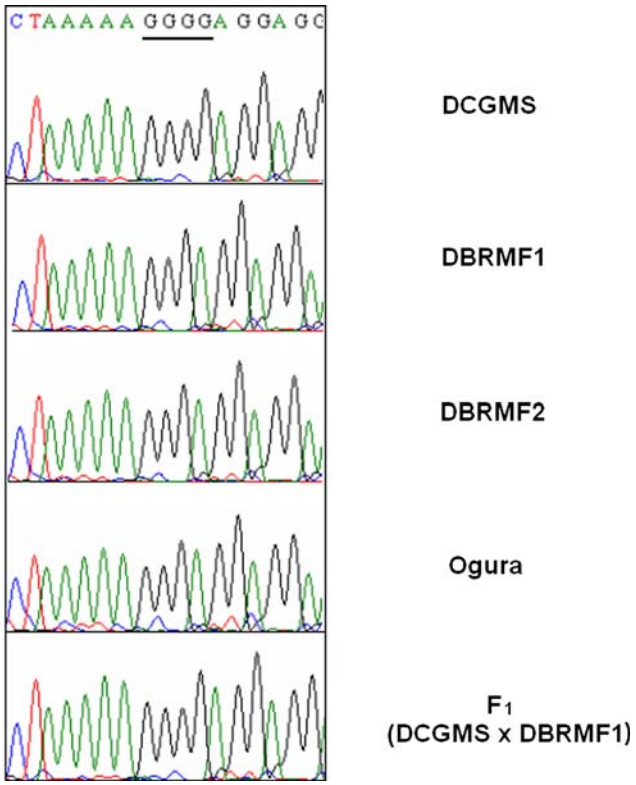

Fig. 5 DCGMS-specific SNP marker detection by direct sequencing. The poly G adjacent to the SNP was underlined. The F_1 hybrid originated from the cross between a DCGMS line as a female parent and a DBRMF1-containing male parent

Discussion

Short repeat sequence-mediated radish mtDNA rearrangement

In the previous study (Lee et al. 2008), a novel cytoplasmic male sterility and mitotype were reported in radishes. This new mitotype shows a different PCR amplification profile from the three previously reported radish mitotypes (Ogura, DBRMF1, and DBRMF2). The most conspicuous feature of the PCR amplification pattern of the DCGMS mitotype is the absence of PCR products containing the *atp6* gene. Since the *atp6* gene is known to be an indispensable gene for mitochondrial function, we assumed that *atp6* in the DCGMS mitotype must have a different contextual organization compared to the other three mitotypes. Indeed, the *atp6* gene in the DCGMS mitotype was revealed to be linked to the *nad3* and *rps12* genes. Through scrutiny of the new *atp6–nad3–rps12* unit, we found that the same short repeat cluster which was involved in the double recombination that produced the distinction between the DBRMF1 and DBRMF2 mitotypes (Kim et al. 2007), also participated in the recombination event that produced the new gene organization. The absence of the second recombination event which occurred between the R5 and R6 repeats of DBRMF1 and DBRMF2 (Fig. 2), led to creation of a new gene organization in the DCGMS mitotype.

Few studies have presented the specific evidence of the involvement of short repeat sequences in mtDNA rearrangements, although several reports have emphasized their importance in mitochondrial genome rearrangement (Small et al. 1989; Fauron et al. 1990; Rankin et al. 1996). We have demonstrated that the R3–R6 short repeat cluster resided in the coding sequence of the chimeric gene, *orf263*, responsible for alloplasmic cytoplasmic male sterility in *Brassica* lines, and that the cluster was also involved in generating a new chimeric organization consisting of the 5′ part of *coxI* gene and the 3′ part of *orf263* gene (Fig. 3). Interestingly, the R3 repeat contains the start codons of both the radish *coxI* gene and the *Brassica tornefortii orf263* gene. It is possible that translocation of the R3 repeat together with the adjacent *atp6* promoter into other mitochondrial genomic regions through mtDNA rearrangements may create new active chimeric genes. Indeed, the widespread abundance of short repeat sequences throughout the radish mitochondrial genome is likely a driving force in mtDNA shuffling.

The active involvement of short repeats in mtDNA rearrangements presented in this study appears to have begun in

the common ancestor of the Brassicaceae family. This is evidenced by the finding that the short repeats are found throughout the Brassicaceae family, whereas the complete mitochondrial genomes of tobacco, rice, and sugar beet reveal at most a single homologous sequence if any at all (Table 2). This result implies that plant mitochondrial genomes may have diverse short repeats that are unique to their own species or family, and the analysis of the repeated sequences of the complete tobacco mtDNA (Sugiyama et al. 2005) supports this hypothesis. According to their divergence time estimates, the radish repeat cluster (R2–R6) might have been active as much as 17–18 million years ago in the common ancestor of *Brassica* and *Arabidopsis* (Bowers et al. 2003; Yang et al. 2006). A high copy number of the R2 repeat in *Brassica napus* is of interest and suggests that this repeat might have recently multiplied in the *Brassica* lineage. Therefore, systematic analysis of short repeat sequences distributed in plant mitochondrial genomes may provide a useful means of identifying candidate chimeric genes that affect agronomically important traits such as male sterility.

Identification of a novel truncated *atp6* gene organization

An additional novel gene organization consisting of a 5′ partial sequence of the *atp6* gene linked to a sequence of unknown origin was identified by genome walking designed to isolate the flanking regions of *atp6* in the DCGMS mitotype. The 3′ unknown sequence had no significantly similar sequences of mitochondrial origin, but a somewhat similar sequence (73% homology) was identified from nuclear genome of *Arabidopsis*. Through the analysis of the F_1 and BC_1F_1 populations using a SNP marker in the truncated *atp6* region, we confirmed the maternal inheritance of this DNA unit. Therefore, this unknown sequence is likely to have been acquired from the nuclear genome and inserted into the *atp6* gene. Frequent DNA transport between the nuclear and mitochondrial genomes during evolution of higher plants has been well documented (Notsu et al. 2002). The transport of this unknown sequence form the nuclear genome seems to have happened relatively recently in radishes since no homologous sequences were identified in the *Brassica napus* and *Arabidopsis* mtDNA. In addition, this organizational unit appears to be a substoichiometric molecule in the mitochondrial genome. Together, these data suggest that this mtDNA fragment originated as a small fragment of radish nuclear genome that was transported into the mitochondria and incorporated into substoichiometric mtDNA molecules. This substoichiometric mtDNA intermediate could eventually be positioned on the master circle via genomic shifting or further rearrangement according to the three-stage model of mtDNA reorganization suggested by Small et al. (1989).

One intriguing feature of the truncated *atp6* gene organization that we identified in this study is that no homologous short repeat sequence was found around the junction between the *atp6* gene and the unidentified sequences at its 3′ end. On the contrary, other rearrangements reported in this study were always accompanied by short repeat sequences flanking the breakpoints. This indicates that other unidentified short repeats may exist in the radish mitochondrial genome, or that other unknown mechanisms accelerating mtDNA rearrangement may have produced this chimeric gene.

Utilization of the SNP marker in the identification of the DCGMS mitotype

A single nucleotide insertion was identified in the *atp6* coding sequence of the truncated *atp6* fragment (Fig. 4). The insertion of a single nucleotide causes frame-shifting, and therefore, if a protein is produced from transcripts derived from this locus, it would differ considerably from the canonical ATP6 protein. Although the level of expression is very low, the existence of the transcript was confirmed by RACE PCR. Unlike the other three mitotypes containing no insertion mutation, the DCGMS mitotype may produce a unique protein product. Therefore, this protein may be the product of a candidate male-sterility inducing gene; however, no effect of restorer-of-fertility gene(s) on the expression of this novel *orf225* was observed. Of course, further analysis of post-transcriptional events is necessary to determine the role of *orf225* on the male-sterility induction. Nonetheless, this SNP can be utilized as a molecular marker that allows for detection of the DCGMS mitotype, and our comprehensive analysis of radish germplasm proved its effectiveness in the detection of the DCGMS mitotype. Interestingly, the recently reported NWB radish CMS (Nahm et al. 2005), whose male-sterile phenotypes were similar to those of DCGMS male-sterile lines, was revealed to contain no insertion mutation like the other three mitotypes (data not shown). In contrast to other molecular makers for radish mitotype classification (Kim et al. 2007) in which the presence or absence of PCR products is affected by the stoichiometry of the mtDNA, this SNP marker is more reliable because it is based on a stable nucleotide change in the coding sequence of the *orf225*. However, a method of SNP detection more efficient than direct sequencing is needed for large-scale screening of samples.

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