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ORIGINAL PAPER

Identification of a novel mitochondrial genome type and development of molecular markers for cytoplasm classification in radish (*Raphanus sativus* L.)

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Abstract Plant mitochondrial genomes have complex configurations resulting from the multipartite structures and highly rearranged substoichiometric molecules created by repetitive sequences. To expedite the reliable classification of the diverse radish (*Raphanus sativus* L.) cytoplasmic types, we have developed consistent molecular markers within their complex mitochondrial genomes. *orf138*, a gene responsible for Ogura male-sterility, was detected in normal cultivars in the form of low-copy-number substoichiometric molecules. In addition to the dominant *orf138-atp8* Ogura mitochondrial DNA (mtDNA) organization, three novel substoichiometric organizations linked to the *atp8* gene were identified in this study. PCR amplification profiles of seven *atp8*- and *atp6*-linked sequences were divided into three groups. Interestingly, the normal cytoplasm type, which had previously been considered a single group, showed two patterns by PCR amplification. The most prominent difference between the two normal mtDNAs was size variation within four short-repeat sequences linked to the *atp6* gene. This variation appeared to be the result of a double crossover, mediated by these homologous, short-repeat sequences. Specific PCR amplification profiles reflecting the stoichiometry of different mtDNA fragments were conserved within cultivars and across generations. Therefore, the specific sequences detected in these profiles were used as molecular markers for the classification of diverse radish germplasm. Using this classification system, a total of 90 radish cultivars, or accessions, were successfully assigned to three different mitotypes.

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Introduction

Mitochondria play an essential role in energy production in plants, animals, and fungi. In higher plants, these organelles have peculiar genome structural features. The size of their genomes range from 208 kb in *Brassica hirta* to over 2,400 kb in some members of the Cucurbitaceae family (Ward et al. 1981; Palmer and Herbon 1987). The monocircular mitochondrial genome found in *B. hirta* (Palmer and Herbon 1987) is considered to be uncommon (Oldenburg and Bendich 2001; Knoop 2004), given the newly completed sequencing of the circular master mitochondrial genomes of *Arabidopsis* (Unseld et al. 1997), rice (Notsu et al. 2002), sugar beet (Kubo et al. 2000), and rapeseed (Handa 2003).

Repeat sequence-mediated recombination contributes substantially to the complex organization of plant mitochondrial genomes. There are two known types of repeats in plant mitochondrial DNA (mtDNA). Large repeats, including the 2.4-kb repeats in *B. napus* (Handa 2003) and

the 6.2-kb repeats in sugar beet (Kubo et al. 2000), contribute to the formation of multipartite mtDNA structures via frequent repeat-mediated recombination (Klein et al. 1994; Albert et al. 1998). Short repeats include those in sugar beet mtDNA, which contain 72 short-repeat sequences that vary in size from 50 to 626 bp (Kubo et al. 2000). Recombination mediated by these short repeats is thought to be a major factor in mitochondrial genome rearrangement, an infrequent but important component of mtDNA evolution (Albert et al. 1998). Variations in mitochondrial genome organization are so common that they can be observed even within the same species (Palmer 1988; Ullrich et al. 1997).

Mitochondrial DNA rearrangement may be accompanied by variations in the stoichiometry of the newly rearranged mtDNAs. This stoichiometry may change rapidly through the process of genomic shifting (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. This process can be activated by stresses such as tissue culture (Fauron et al. 1990) or somatic hybrid regeneration (Sakai and Imamura 1992; Motegi et al. 2003) and is controlled by nuclear genes including the *Fr* gene in *Phaseolus vulgaris* (Mackenzie and Chase 1990; Janska et al. 1998) and the *CHM* gene in *Arabidopsis* (Abdelnoor et al. 2003).

Genomic shifting often leads to maternally inherited phenotypic changes by either increasing or decreasing the expression of genes encoded by substoichiometric mtDNA through a process that involves increasing or decreasing the copy number of such genes. Cytoplasmic male-sterility (CMS) is the best-known trait induced by stoichiometric shifting (Janska et al. 1998; Motegi et al. 2003), though direct sterility-inducing factors have been shown to be chimeric genes present in mtDNA (Hanson 1991). These novel chimeric genes are also thought to be created through dynamic, short-repeat sequence-mediated mtDNA rearrangement (Hanson and Bentolila 2004).

Ogura CMS (Ogura 1968) is the most extensively studied type of male-sterility currently used in commercial radish F_1 hybrid production. The Ogura CMS has been introduced into a variety of *Brassica* species through either conventional breeding (Bannerot et al. 1974; Paulmann and Röbbelen 1988) or protoplast fusion (Pelletier et al. 1983; Walters et al. 1992). Because of its stable male-sterility and simple restoration of fertility, Ogura CMS has been used in the development of hybrid varieties. A novel mitochondrial gene, *orf138*, was identified as being responsible for Ogura CMS (Bonhomme et al. 1991; Grelon et al. 1994). Additionally, its nuclear restorer-of-fertility gene, which encodes a pentatricopeptide repeat (PPR) protein, has recently been cloned (Brown et al. 2003; Desloire et al. 2003; Koizuka et al. 2003). In an effort to identify the Ogura CMS-inducing gene, highly variable rearranged mtDNAs in Ogura and normal radishes were identified. In this case, the comparison has been limited to only a single type of normal radishes (Makaroff and Palmer 1988; Makaroff et al. 1989; Krishnasamy and Makaroff 1993). Previous studies focusing on the identification of radish cytoplasm variants have been reported (Yamagishi and Terachi 1994, 1996, 2001), but the classification system used in these studies relied on dichotomy (Ogura or normal) because the molecular markers for cytoplasm distinction were based on a limited number of mtDNA regions and because the stoichiometry of mtDNA was not considered.

In the present study, a novel mtDNA organization of radish cytoplasm was identified in normal radish cultivars. We observed vivid examples of radish mtDNA rearrangement caused by ubiquitous short-repeat sequences. Furthermore, we developed a new combination of molecular markers that can be used for the reliable classification of radish cytoplasms.

Materials and methods

Plant materials

A total of 90 radish germplasms, consisting of 56 commercial cultivars and 34 accessions, were used as plant materials in this study (supplementary Table 1). Most of the commercial cultivars were bred by Korean seed companies, with some accessions introduced from China and Tibet. Four plants of each line were germinated in 105-cell plug trays measuring 54 × 28 × 8 cm. At the two-leaf-stage, seedlings were placed in a cold chamber (4°C) for 40 days to promote vernalization. After cold treatment, the seedlings were grown in the greenhouse, and male-sterility was determined by visual examination.

Two populations, consisting of four consecutive generations produced by artificial self-pollination following a SSD (Single Seed Descent) method, were used to test conservation of PCR amplification profiles in the mtDNA fragments across generations.

DNA extraction and PCR amplification

Total genomic DNA was extracted from the leaf tissue of three-leaf stage radishes using the DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol. PCR was performed in a 10-μl reaction mixture containing 0.05-μg of template, 0.1 μl of 10× PCR buffer, 0.2 μl of forward primer (10 μM), 0.2 μl of reverse primer (10 μM), 0.2 μl of dNTPs (10 mM each), and 0.1 μl of Advantage 2 Polymerase Mix (Clontech, Palo Alto, CA, USA). PCR 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 to amplify eight mtDNAs are presented in Table 1.

Sequencing of the PCR products

Following successful PCR amplification (as determined by the presence of a single band on a 1% agarose gel), the PCR products were purified using the QIAquick PCR Purification kit (QIAGEN, Valencia, CA, USA). The purified PCR products were sequenced either directly or after 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, Foster City, CA, USA).

Genome walking for the determination of sequences linked to the *atp8* (*orfB*) gene

Since the product of the *orfB* gene was identified as a subunit of ATP synthase (Sabar et al. 2003), a known homologue of yeast and mammalian *ATP8*, the *orfB* gene will be referred to as *atp8* for the remainder of this report. To identify sequences linked to the *atp8* gene, genome walking was performed using the Universal GenomeWalker Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol. Genome walking libraries were constructed from the genomic DNA of a breeding line containing Ogura cytoplasm. Gene-specific primers were designed based on the coding sequence of the *atp8* gene.

Results

Amplification of the *orf138* gene from normal and Ogura radishes

Previously, molecular markers for the specific amplification of the *atp6*, *coxI* and *orf138* genes had been used to classify diverse radish germplasm based on cytoplasmic type (Yamagishi and Terachi 1994, 1996). However, we found that Ogura-specific bands appeared in some cultivars with cytoplasm types that were predicted to be normal according to the cultivar pedigree, although at low intensity (data not shown). Conversely, the normal cultivar-specific bands also appeared at low intensity in some Ogura CMS cultivars. The identities of the unexpected bands were confirmed by sequencing. Since the reported molecular markers were dominant markers, such that the marker haplotypes were identified by the presence or absence of the specific PCR products, the presence of the light-intensity Ogura-specific band in normal radishes may result in the mis-classification of radish germplasm.

To test whether the appearance of low-intensity bands was reproducible, additional primers flanking the *orf138* and *coxI* genes were used for PCR amplification. The results confirmed the existence of the *orf138* sequences in normal radishes (Fig. 1). Moreover, one higher molecular weight band was amplified in normal radish only when the primer combinations of 2 + 3 and 2 + 4 were used in PCR amplification. Repeated PCR amplification with slightly modified annealing temperatures proved highly reproducible. The identity of the high molecular weight band was determined, by sequencing, to be the full-length *atp6* gene

Table 1 Primer sequences used for amplification of the eight mtDNA fragments in Fig. 2

Molecules	Primer names	Primer sequences (5′–3′)
1	orf138-F	GTAGCCCTGTATGGACTCGCGAAGCAG
	orf138-R	CCAGCTTAGCCAAATATGGGCCATTAG
2	cox1-F	GTCATTCCAGGTCCACGCATGTTGAAG
	atp8-R	TGCTGATCCCAAGTACTCCATCTCCATCA
3	atp6-F1	CCTCCCCTCAAGCACGACAGATTGACT
	orf138-R	CCAGCTTAGCCAAATATGGGCCATTAG
4	atp6-F2	AAGTGGGTTCGCTTGGACTATGCTATG
	atp8-R	TGCTGATCCCAAGTACTCCATCTCCATCA
5	stu-F	GAGGAATCACAGAGAATGCCCTGCACA
	atp8-R	TGCTGATCCCAAGTACTCCATCTCCATCA
6	atp6-F2	AAGTGGGTTCGCTTGGACTATGCTATG
	atp6-R1	GCCTGTTCCCTCTGAATCGGTTGATTG
7	atp6-F3	GAAGCAGCAGATAGCCACACCACAGGA
	atp6-R2	AGCAGCATGAACAAAGATGGATTTGTGAA
8	Nad3-F	AATGTGGTTTCGATCCTTCCGGTGATG
	Rps12-R	CTGCACCATATTTGGATCTGCCGCTTC

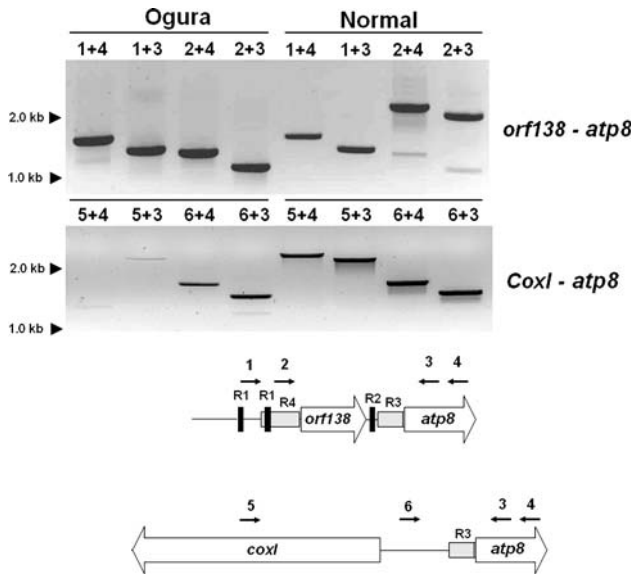

Fig. 1 PCR amplification of the Ogura- and normal-specific sequences using different combinations of primers based on the coding and flanking sequences of the *orf138* and *coxI* genes. *Arrow-shaped boxes* indicate the 5′–3′ direction. The *rectangular*, *gray boxes* indicate short-repeat sequences. *R1–R4* short repeats. *Arrows* indicate the binding sites of primers

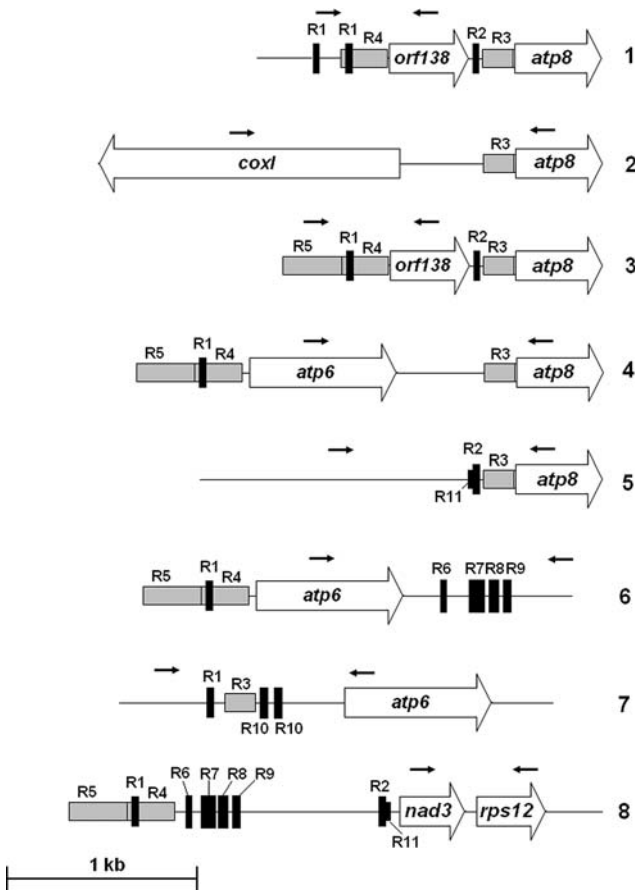

Fig. 2 Organization of the mitochondrial genome fragments used in this study to develop molecular markers for radish cytoplasm classification. *Arrow-shaped boxes* indicate the 5′–3′ direction. The *rectangular*, *gray boxes* indicate short-repeat sequences. *Arrows* indicate the binding sites of primers used for specific amplification of each fragment. *R1–R11* short repeats. *1–8*: numbers assigned to each fragment referenced in the text

linked to the *atp8* gene (Molecule 4 in Fig. 2). The sequence revealed that the *orf138* in Molecule 1 and *atp6* gene in Molecule 4 were flanked by the same sequences, which is the coding sequence of the *atp8* gene and a repeat sequence (R4). This arrangement allowed the two bands to be amplified from normal radishes since the primer 2 had been based on the repeat sequence (R4).

The linked *atp6–atp8* has not been reported previously. This newly described organization may be the result of short-repeat sequence-mediated rearrangement in the radish mitochondrial genome. While determining the effect that this organization has on the function of radish mitochondria is beyond the scope of this study, the existence of variations in the flanking sequences of the *orf138* and *atp6* genes may prove to be a complicating factor in the development of reliable molecular markers for classification of radish germplasm.

Identification of novel gene organizations linked to the *atp8* gene

To further explore the organization of the genome proximal to the *atp8* gene, genome walking was performed using a library constructed from the DNA of an Ogura CMS breeding line. Two additional gene organizations linked to the *atp8* gene were identified. One of these (Molecule 3 in Fig. 2) was the *orf138–atp8* organization, which was identical to the previously reported sequence (Molecule 1, Krishnasamy and Makaroff 1993) except for 5′ repeat sequences (R5-R1-R4 in Molecule 3; R1-R1-R4 in Molecule 1). These 5′ repeat sequences were identical to those previously reported for the normal *atp6* gene (Molecule 6, Krishnasamy and Makaroff 1993) and the newly identified *atp6–atp8* organization (Molecule 4).

Another sequence obtained by the genome walking was a mitochondrial intergenic sequence linked to the *atp8* gene (Molecule 5). The 862-bp intergenic sequence shared 98–99% nucleotide sequence identity with sequences identified in the *Brassica* species. In three different *Brassica* species, this sequence was linked to the *atpA* gene (Fig. 3). Interestingly, the short-repeat sequences were present in *B. juncea* and *B. napus*, but absent in *B. rapa*, suggesting that they may have existed in a common ancestor of *Raphanus* and other *Brassica* species. This hypothesis is supported by the finding that dynamic short-repeat sequence-mediated gene rearrangement is commonly observed in the family Brassicaceae.

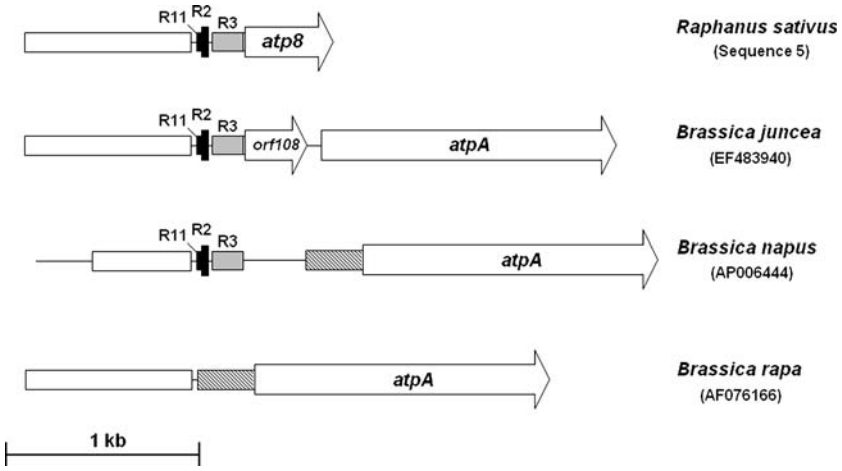

Fig. 3 Comparison of homologous mitochondrial gene organizations isolated from other *Brassica* species with the unique sequence present in Molecule 5 that is shown in Fig. 2. The *empty boxes* indicate the sequences homologous to the unique sequence isolated from radish by genome walking. *Arrow-shaped boxes* indicate the 5′–3′ direction. The *filled*, *gray*, and *hatched rectangular boxes* indicate respective homologous sequences. *R2, R3, R11* short repeats

Assessment of novel gene organization copy number in diverse radish germplasm

Previously, the *orf138* gene, a male-sterility inducing gene in Ogura CMS radish, was detected in normal radishes in the form of low-intensity PCR-amplified bands. These low-intensity bands probably resulted from the low copy number of the *orf138* gene in normal radishes. The eight sequences shown in Fig. 2, including the newly discovered gene organizations identified from the genome walking experiments described above, were amplified from both normal and Ogura radishes using varying numbers of PCR cycles. The sequences assumed to be present in dominant mitochondrial genomes [according to previous reports (Makaroff et al. 1989; Krishnasamy and Makaroff 1993)] appeared as distinct bands in 30 cycles of PCR. However, the sequences that may be present in substoichiometric subgenomes were either absent or appeared only as faint bands in 30 cycles of PCR or as either faint or intense bands in 40 cycles (data not shown). The band pattern that resulted from the eight sequences observed in normal and Ogura radishes was reproducible when additional individuals, some representing specific cultivars, were tested (Fig. 4). Furthermore, the PCR amplification profiles of eight mtDNA fragments were conserved across four consecutive generations produced by artificial self-pollination (Fig. 5).

Defining a new normal mitotype and the classification of radish germplasm using a combination of molecular markers

When PCR examination of the eight sequences was performed for a large number of radish cultivars and accessions, the band patterns resulting from the eight sequences were consistent across cultivars containing Ogura cytoplasm, although the band intensity of the substoichiometric sequences was slightly variable. However, the band

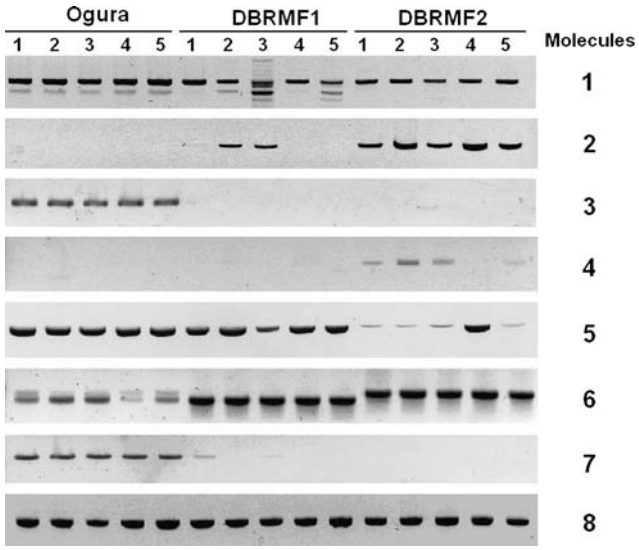

Fig. 4 Comparison of the PCR amplification profiles for three types of radish cytoplasm. *1–5, 6–10, 11–15* representative cultivars for each mitotype. *Fragment 1–8* mtDNA fragments from Fig. 2

patterns observed for the normal radishes could be classified into two distinct groups (Fig. 4). The most conspicuous difference between these two groups, which represented normal samples, was observed for Molecule 6 marker. For this marker, an approximately 835 bp band was observed in one group, while an approximately 910 bp band was observed in the other (Fig. 4). The group containing the 910-bp band represented the normal cytoplasm classified in the previous reports (Makaroff et al. 1989; Krishnasamy and Makaroff 1993) because the normal-specific sequence (Molecule 2) was strongly amplified. Hereafter, this previously reported (Makaroff et al. 1989; Krishnasamy and Makaroff 1993), normal mitochondrial genome variant (mitotype) is designated DBRMF2, and the other mitotype, which produced the 835-bp band, is designated DBRMF1. As with Molecule 8, both sized bands are present as a

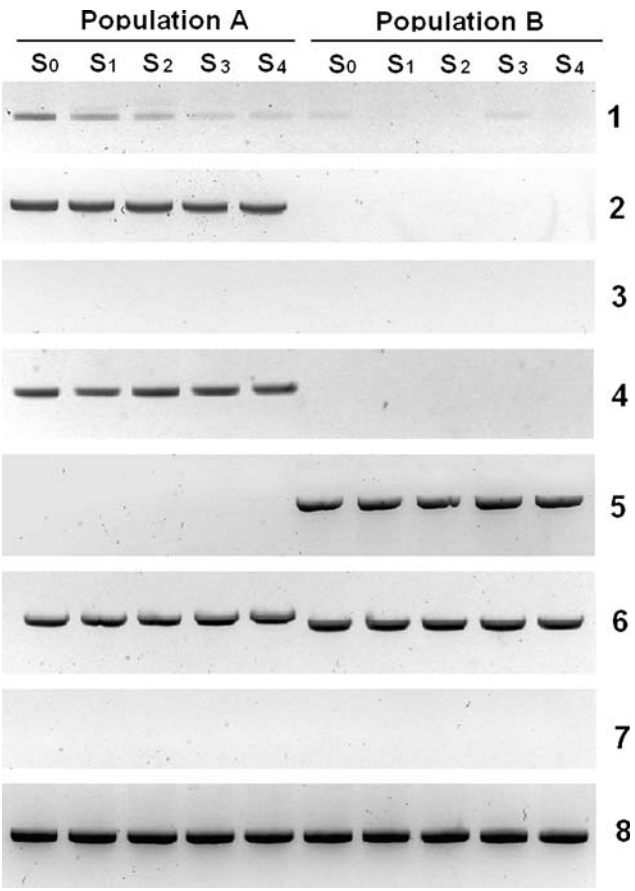

Fig. 5 Conserved stoichiometry of mtDNA fragments across generations. *S0–S4* generations of SSD (Single Seed Descent) populations. *Fragment 1–8* mtDNA fragments from Fig. 2

dominant mtDNA because they were detected as intense bands after only 30 cycles of PCR amplification. Sequencing of these two PCR products revealed that they both contain the same short-repeat sequences from R6 to R9, but they have different-sized spacers between these short-repeat sequences (Fig. 6a). Sequence analysis revealed that an array of short-repeat sequences (R6-R8) in the DBRMF2 was replaced by a homologous sequence that was linked to the *nad3* and *rps12* genes (Molecule 8). Apparently, the 69 and 6-bp deletions were responsible for the size difference. It is possible that this replacement might arise from a double recombination event between the repeat sequences in Molecules 6 and 8 (Fig. 6b). Interestingly, the 910 and 835-bp PCR products were simultaneously detected in the Ogura mitotype; however, the intensity of the two bands was low, suggesting that they are present as substoichiometric mtDNAs.

Using the combination of molecular markers developed in this study on the basis of the eight mitochondrial sequences, a total of 90 cultivars or introduced accessions were successfully classified. The results showed that the newly identified DBRMF1 mitotype was prevalent in diverse cultivars and introduced accessions. This finding indicates that mitotype DBRMF1 appeared over a long evolutionary time period since it is not a rarely distributed variant generated through a recent mitochondrial genome rearrangement (Table 2).

Discussion

Complex organization of the radish mitochondrial genome

The present study describes evidence of highly variable, rearranged mtDNA in radish. Our findings are consistent with observations from previous studies showing extensive mtDNA rearrangement in Ogura and normal cytoplasm (Makaroff and Palmer 1988). Each of the two dominant, differently sized arrays of short-repeat sequences within Molecule 6 (Fig. 2), which were likely generated via short-repeat sequence-mediated double recombination, were present in two different radish mitotypes. Although these two sequences were present together in most Ogura cytoplasms, the copy number of each marker was significantly reduced to the substoichiometric level. Co-existence of both sequences in Ogura mtDNA may be an indication of a transitional stage occurring before either of the two sequences formed their dominant organization. This hypothesis supports the three-stage model for mtDNA reorganization involving substoichiometric intermediates that was previously proposed by Small et al. (1989) as being prevalent in higher plant species. Such a three-stage model provides an explanation for how the diversity and complexity of higher plant mitochondrial genomes can be achieved.

In addition to size variation in a key marker (Molecule 6), we identify three other new genomic organizations linked to the *atp8* gene. All of these organizations likely exist at the substoichiometric level. One of these *atp8*-linked organization was predominant within a radish mitochondrial genome, while the others were only observed at the substoichiometric level. The diverse organizations created by short-repeat mediated rearrangement may serve as a reservoir for diversity that is used in the rapid evolution of the mitochondrial genome of radish. Although the exact configurations of diverse, rearranged fragments identified in this study were not entirely resolved, our results provide a good example of the role that short-repeat sequences can play as a driving force for mtDNA evolution.

Maintenance of stoichiometry in subgenomic mtDNAs

The relative copy number of the new sequence organizations described in this study appeared to be conserved within radish mitotypes and across generations. This finding indicates that there may be an active mechanism that

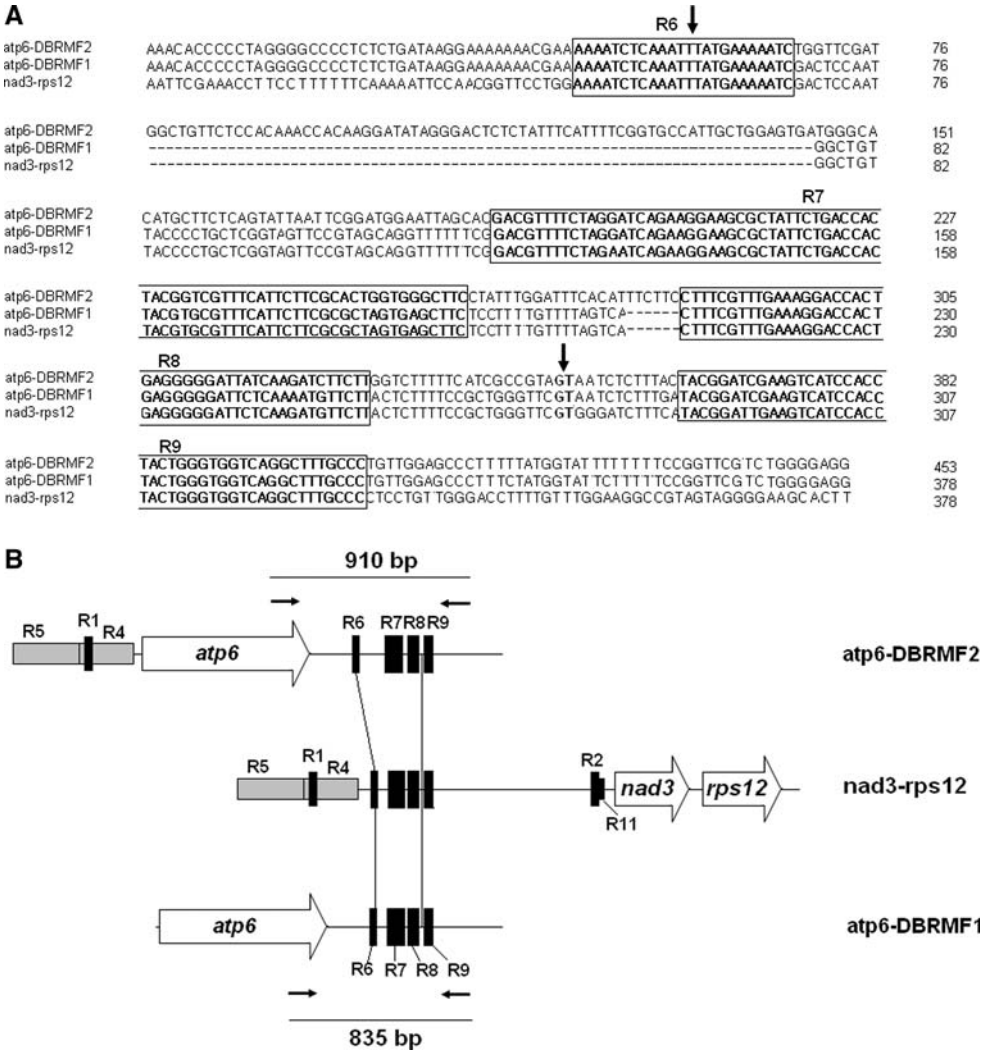

Fig. 6 Comparison of the array of short-repeat sequences residing in Molecule 6 of DBRMF1 and DBRMF2 mitotypes. **a** Alignment of nucleotide sequences of three homologous repeat sequences. *Arrows* indicate the putative breakpoints of crossovers. Nucleotide sequences in the *rectangular boxes* represent short repeats. **b** Structure showing short-repeat sequence-mediated recombinations. *Arrow-shaped boxes* indicate the 5′–3′ direction. The *rectangular boxes* indicate short repeat sequences. *Arrows* indicate the binding sites of primers used for specific amplification of different-sized arrays of short-repeat sequences

Table 2 Distribution of three different mitotypes in diverse radish germplasm

Types of germplasm	Mitotypes			
	Ogura	DBRMF1	DBRMF2	Total
Accessions	13	18	3	34
Cultivars	18	20	18	56
Total	31	38	21	90

maintains the stoichiometry of subgenomes. Previous studies have reported evidence suggestive of the active maintenance of stoichiometry across generations in *Brassica* cybrids (Sakai and Imamura 1993; Bellaoui et al. 1998). Nonetheless, how these low-copy-number mtDNA molecules, which may be present in less than one copy per 100 cells of vegetative tissues (Arrieta-Montiel et al. 2001), can be transmitted from one generation to the next without frequent loss is still enigmatic. Arrieta-Montiel et al. (2001) explained this phenomenon by suggesting a model in which low-copy-number molecules might be transmitted to progeny through a master chromosome that contains all mtDNA molecules and that this master circle may be present in meristemic tissues. This model is supported by the finding that the copy number of the substoichiometric molecules is significantly increased in meristemic tissues relative to vegetative tissues.

Once transmitted to the next generation, this master circle might be divided into multipartite subgenomes, and the copy number of each subgenome will be determined by a monitoring program. Although the identity of such a monitoring program is still unknown, it could be regulated by genetic factors or environmental stimuli. The *Fr* gene in common bean (Mackenzie and Chase 1990; Janska et al. 1998) and the *CHM* gene in *Arabidopsis* (Abdelnoor et al. 2003) are nuclear loci known to control genomic shifting. As to environmental stimuli, genomic shifting induced by tissue culture (Fauron et al. 1990) is mainly reported, but X-ray irradiation (Chung 1974) or crossing with distantly related cultivars (Chung et al. 1977) produced CMS in

radish. Induced male-sterilities are proposed to be the result of genomic shifting rather than mutation because the phenotype and inheritance of this trait is similar to that of Ogura male-sterility (personal communication).

Taken together, the existence of a variety of rearranged substoichiometric mtDNA molecules, their transmission to the next generation, and their amplification by genomic shifting reflect the high-evolutionary plasticity of plant mitochondrial genomes.

Identification of a new radish mitotype and development of molecular markers for radish cytoplasm classification

Since the first report of Ogura male-sterile radish cytoplasm (Ogura 1968), the comparison of mitochondrial genomes in radish has been limited to only two types of mtDNAs: Ogura and normal cytoplasms. This type of classification indicates a single type of normal cytoplasm in radishes. However, we have observed that normal cytoplasms could be further divided. A novel radish mitotype was identified in a large proportion of accessions and cultivars in this study. With the exception of the unusual Molecule 6 marker that is the result of a double crossover, the stoichiometry of the various subgenomic mtDNA significantly varied between the two normal mitotypes. Similarly, two types of mitochondrial genomes (NA and NB), between which genome organizations were highly rearranged, were identified in maize and the cmsT cytoplasm (Fauron et al. 1990).

Known phenotypic outcomes induced by new mitochondrial genomes such as male-sterility resulting from the coexistence of the male-sterility-inducing mitochondrial gene and the homozygous recessive nuclear restorer-of-fertility gene, are still being investigated. It is possible that there may be unidentified incompatibility between a new mitochondrial genome and some nuclear genes present in distantly related radish germplasm.

Molecular markers used for radish cytoplasm classification have previously been used to distinguish between Ogura and one type of normal cytoplasm (Yamagishi and Terachi 1994, 1996, 2001). These markers were dominant so that each haplotype could be determined based on the presence or absence of the specific PCR products. We found that, in the case of dominant markers, specific PCR products could mistakenly be amplified from low-copy-number substoichiometric mtDNAs depending on PCR conditions or template concentrations.

A combination of molecular markers developed in this study has been used to classify radish cytoplasms into three groups. Only the marker based on Molecule 6 can be used to classify all three of the radish mitotypes that were examined here. Furthermore, it is important to mention that the fact that this marker is a co-dominant rather than a dominant marker and will prove to be a very useful tool for radish breeding and germplasm evaluation.

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