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ORIGINAL PAPER

Discovery of a novel cytoplasmic male-sterility and its restorer lines in radish (*Raphanus sativus* L.)

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Abstract A male-sterile (MS) radish (*Raphanus sativus* L.) was found in an accession collected from Uzbekistan. Unlike Ogura MS radishes in which no pollen grain is typically visible during anthesis, a small number of pollen grains stuck together in the dehiscing anthers was observed in the newly identified MS radish. Fluorescein diacetate tests and scanning electron micrographs showed that pollen grains in the new MS radish were severely deformed and non-viable. Cytological examination of pollen development stages showed a clear difference in the defective stage from that seen in Ogura male-sterility. Reciprocal cross-pollination with diverse male-fertile lines indicated that pollen grains of the new MS radish were completely sterile, and the female organs were fully fertile. When the new MS radish and Ogura MS lines were cross-pollinated with a set of eight breeding lines, all F_1 progeny originating from crosses with the new MS radish were male-sterile. In contrast, most of the F_1 progeny resulting from crosses with Ogura MS lines were male-fertile. These results demonstrated that factors associated with induction of the newly identified male-sterility are different from those of Ogura male-sterility. The lack of restorer lines for the newly identified male-sterility led us to predict that it might be a complete cytoplasmic male-sterility without restorer-of-fertility genes in nuclear genomes. However, cross-pollination with more diverse radish germplasm identified one accession introduced from Russia that could completely restore fertility, proving the existence of restorer-of-fertility gene(s) for the new male-sterility. Meanwhile, the PCR amplification profile of molecular markers for the classification of radish mitochondrial genome types revealed that the new MS radish contained a novel mitotype.

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Introduction

Cytoplasmic male-sterility (CMS) results in the inability of plants to produce viable pollens grains, and is maternally inherited since CMS-inducting factors reside on mitochondrial genomes. Cytoplasmic male-sterility has been discovered in many plant species including economically important crops such as maize, sorghum, and rice (Hanson 1991). Jones and Clarke (1943) first demonstrated the inheritance pattern of CMS in onions and its restoration by a nuclear gene. Since they also showed that CMS could be successfully applied to F_1 hybrid production, it has been used to develop F_1 hybrid cultivars in many crops.

Cytoplasmic male-sterility has also played an important role in studying the interaction between mitochondrial and nuclear genomes (Schnable and Wise 1998; Budar et al. 2003; Hanson and Bentolila 2004). All CMS reported so far are induced by chimeric genes in mitochondrial genomes. Unlike animal mitochondrial genomes whose size range from 15 to 18 kb, plant mitochondrial genomes are peculiarly large and varied in size from 208 kb in *Brassica hirta* (Palmer and Herbon 1987) to over 2,400 kb in muskmelon

(Ward et al. 1981). Configuration of plant mitochondrial genomes is still enigmatically complex (Oldenburg and Bendich 2001; Knoop 2004), although some plant mitochondrial genomes are completely sequenced (Unseld et al. 1997; Kubo et al. 2000; Notsu et al. 2002; Handa 2003). The ubiquitous presence of repeat sequences in the mitochondrial genomes is one of the major factors contributing to the complexity of plant mitochondrial genomes. Repeat sequence-mediated recombination yields multipartite structures and subgenomic mtDNA molecules (Palmer 1988; Albert et al. 1998). The stoichiometry of subgenomic mtDNAs varies among species and even within the same species (Sakai and Imamura 1993; Bellaoui et al. 1998; Kim et al. 2007). The stoichiometry of subgenomic mtDNA is not constant, with stoichiometric changes being regulated by a mechanism referred to as genomic shifting (Small et al. 1989; Arrieta-Montiel et al. 2001). Tissue culture (Fauron et al. 1990) or genetic factors such as the *Fr* gene in common beans (Mackenzie and Chase 1990; Janska et al. 1998) or the *CHM* gene in *Arabidopsis* (Abdelnoor et al. 2003) are known to trigger genomic shifting.

Male-sterility inducing factors reported in many plant species are chimeric genes that might be created by recombination between short repeat sequences (Schnable and Wise 1998; Hanson and Bentolila 2004). The male-sterility inducing mechanisms of such chimeric genes are still unclear in all cases, but the action of CMS-inducing genes can be suppressed by nuclear restorer-of-fertility genes. With the exception of the maize *Rf2* gene, which encodes aldehyde dehydrogenase and acts indirectly to suppress *urf13*, the CMS-inducing chimeric gene of maize (Cui et al. 1996), other restorer-of-fertility genes cloned in petunia (Bentolila et al. 2002), radish (Brown et al. 2003; Desloire et al. 2003; Koizuka et al. 2003), and rice (Komori et al. 2004) have all been shown to encode pentatricopeptide repeat (PPR) proteins. Approximately 450 members of the PPR gene family have been identified in *Arabidopsis* (Small and Peeters 2000), but their roles have just began to be unveiled in some species. The roles of PPR genes have been reported in rice chloroplast biogenesis (Gothandam et al. 2005) and in *Arabidopsis* chloroplast RNA editing (Kotera et al. 2005).

Since the discovery of CMS in radishes by Ogura (1968), it has become one of the most extensively studied types of male-sterility. Ogura CMS has been adopted in developing radish F_1 hybrid cultivars. The mitochondrial CMS-inducing gene, *orf138*, has been isolated (Bonhomme et al. 1991; Grelon et al. 1994) and its restorer gene, *Rfo*, in the nuclear genome has been cloned (Brown et al. 2003; Desloire et al. 2003; Koizuka et al. 2003). The potential use of Ogura CMS in F_1 hybrid production has been extended to *Brassica* species. Ogura CMS cytoplasm was initially introduced into cabbages (Bannerot et al. 1974) and cauliflowers (Dickson 1985) by backcrossing to produce male-sterile plants. However, the *Brassica* crops containing Ogura cytoplasm showed unacceptable defects including low-temperature chlorosis and low nectar production (Bannerot et al. 1977). These problems were overcome by protoplast fusion between *Brassica napus* containing Ogura cytoplasm and normal *B. napus* cytoplasm (Pelletier et al. 1983; Menczel et al. 1987; Jarl et al. 1989). Subsequently, male-sterile Pak Choi and Chinese cabbage were developed by introgressing Ogura cytoplasm from *B. napus* produced by protoplast fusion (Heath et al. 1994). The restorer-of-fertility gene, *Rfo*, was also introduced into *B. napus* from radish to produce fertility-restored F_1 hybrid rapeseeds (Pellan-Delourme and Renard 1988; Sakai et al. 1996; Primard-Brisset et al. 2005).

In this study, we report a novel radish CMS line whose male-sterility phenotype, mitochondrial genome variant (mitotype), and restorer lines differed from those of Ogura CMS. The potential application of this novel CMS line in radish breeding programs is discussed.

Materials and methods

Plant materials

The new MS radish was found in an accession collected from Uzbekistan. This accession was seemingly not a pure line, because most plants were fully male-fertile, and morphological traits segregated. The cell line of the original MS plant was deposited to Korean collection for type cultures, and an accession number, KCTC 11101BP, was given by the International Depositary Authority. In addition, seeds of the new MS radishes are available upon request for research purposes only.

Two Ogura MS and 24 male-fertile breeding lines and accessions were used in experiments evaluating male- and female-fertility, and male-fertility restoration of the new MS plant. The mtDNA amplification profiles of the newly identified MS plant was compared to the genomic DNA of nine accessions representing three mitotypes that had been reported in the previous study (Kim et al. 2007).

Fluorescein diacetate (FDA) staining and scanning microscopy

Pollen grains of fully fertile and male-sterile plants were collected from dehiscing anthers and stained with FDA to test pollen viability (Heslop-Harrison and Heslop-Harrison, 1970). After a 5-min incubation at room temperature, pollen grains were examined by fluorescence microscopy (excitation filter 465–495 nm, dichroic mirror 505 nm, barrier filter 515–555 nm, Nikon, Tokyo). For scanning

electron microscopy (SEM) investigations, pollen grains were placed on specimen stubs, sputter-coated with platinum particles, and examined using a scanning electron microscope (JEOL: JSM–6700F, Japan).

Cytological examination of male gametophyte development stages

Different stages of anthers were sampled and fixed overnight in a cold fixative solution (50 mM PIPES buffer; 0.3% *p*-formaldehyde) in a cold chamber set at 4°C. The anthers were dehydrated through a series of differently graded ethanol. Dehydrated anthers were embedded into paraffin and sectioned with a rotary microtome. Serial sections (10 μM in thickness) of anther tissues were mounted on slide glasses and stained with 0.25% toluidine blue O. Sectioned anthers were observed with an optical microscope.

DNA extraction and PCR amplification

Total genomic DNA was extracted from leaf tissues 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, 0.1 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). 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 to amplify eight mtDNA fragments are presented in Kim et al. (2007).

Results

Discovery of a new male-sterile plant from radish germplasm evaluation

While evaluating morphological characteristics of radish germplasm collected from Asian countries, a male-sterile radish plant was identified in a collection from Uzbekistan. The morphological traits, other than male-sterility, were not significantly different from those of normal radishes. In particular, a small number of pollen grains were found inside the anther in an aggregated form (Fig. 1c). The pollen grains barely adhered to fingers when dehiscing anthers were touched. The anthers of normal radishes were completely covered with fluffy pollen grains (Fig. 1a), while the anthers of Ogura CMS plants were totally devoid of pollen grains (Fig. 1b). The presence of a small number of aggregated pollen grains led us to predict that this MS plant was different from Ogura CMS in which pollen grains were completely absent. To verify the pollen viability of this unusual plant, the pollen grain morphology was examined using SEM, and the viability was checked with FDA test. Pollen grains of the new MS plant were small in size and severely deformed, compared to those of normal plants (Fig. 2a, b). They also seemed to be non-viable since no fluorescent pollen grain was detected in the FDA test. Male-sterility of this mutant radish was confirmed by pollination experiments in which pollen grains of male parents were artificially transferred to the stigma of female parents. All plants pollinated with these MS pollen grains failed to set seeds (Table 1). The ability of this MS plant to set seed when pollinated with grains of other normal plants indicated that the female-fertility of this MS plant was normal. Furthermore, no recognizable seed set reduction was observed when these MS plants were used as pollen receivers in the succeeding backcross populations (data not shown).

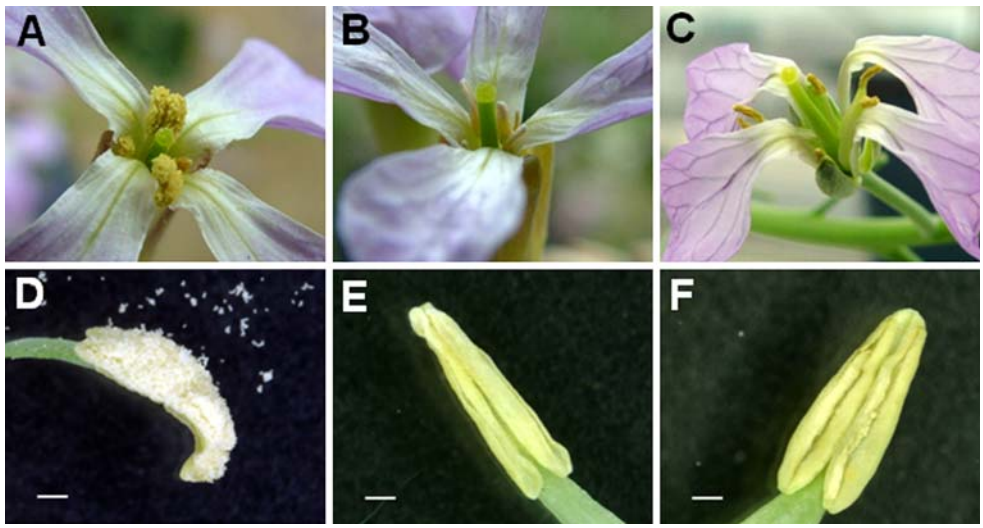

Fig. 1 Radish flowers in full bloom and anthers after pollen dehiscence. **a**, **d** Normal male-fertile radish, **b**, **e** Ogura male-sterile radish, **c**, **f** newly identified male-sterile radish *(bars* indicate 1 mm)

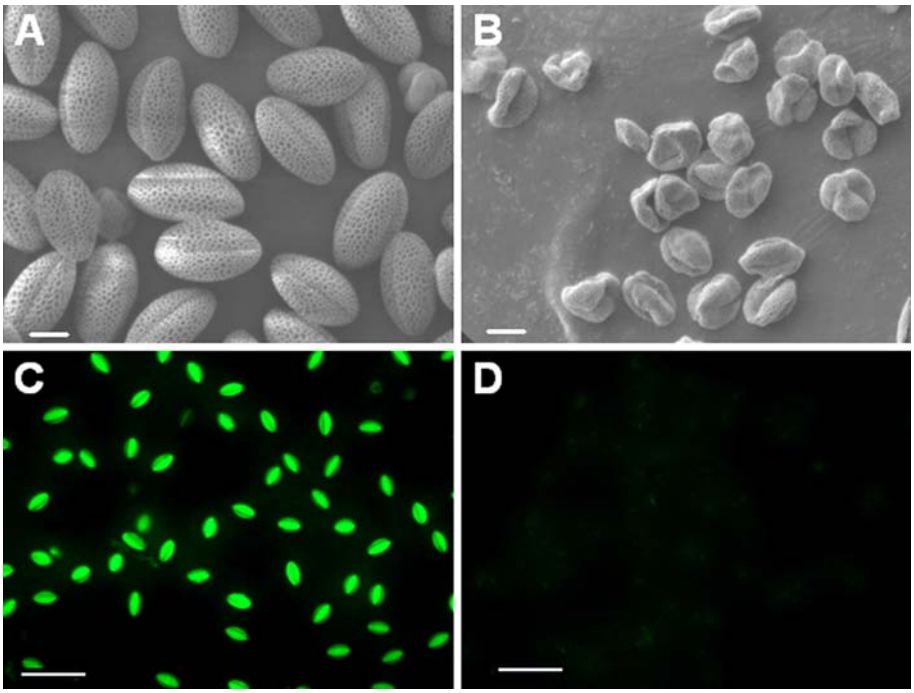

Fig. 2 Morphology and viability of pollen grains of normal and male-sterile radishes. Scanning electron micrographs (**a**, **b**) and fluorescence micrographs of FDA-stained pollen grains (**c**, **d**). **a**, **c** Normal male-fertile radish, **b**, **d** newly identified male-sterile radish [*bars* indicate 10 μm (**a**, **b**) and 100 μm (**c**, **d**)]

Table 1 Fertility tests of the new MS radish by cross-pollination with diverse breeding lines

Female parental lines	Male parental lines				
	New MS	R12	R15	R20	R29
New MS	–	+	+	+	+
R49	–	+	+	+	+
R106	–	+	+	+	+
R122	–	+	+	+	+
R126	–	+	+	+	+
R127	–	+	+	+	+

+, – presence and absence of seed setting after artificial cross-pollination, respectively

Cytological examination of male gametophyte development in the new MS plant

To identify the stage in pollen development at which abnormal pollen abortion occurs and to compare the abortion mechanisms between Ogura CMS and this MS radish, transverse anther sections from early to late stages of pollen development were examined by optical microscopy. Within the pollen mother cell stages, no abnormality was detected among the three radishes (Fig. 3a–c). At the tetrad stage, the tapetum cells of Ogura CMS bulged by severe cytoplasmic vacuolation (Fig. 3e), but no significant differences were observed between normal and new MS plants during this stage (Fig. 3d, f). After the microspores were released, microspores of Ogura CMS began to degenerate (Fig. 3k) finally resulting in empty anthers with no pollen grains (Fig. 3n). The abortion mechanism of the Ogura CMS was consistent with the previous reports (Su et al. 1995). However, the microspores of the new MS plant failed to develop into mature pollen grains. The size of the just-released microspores and the just-before-dehiscence stage microspores remained unchanged. They appeared as a single clump as they stuck together (Fig. 3o) at the stage when mature pollen grains develop in normal radishes (Fig. 3m). Although the cytological examination of microsporogenesis could not pinpoint the exact abortion mechanism of this new MS plant, these results did show that the mechanism of male-sterility in this MS plant was different from that of Ogura male-sterility.

Inheritance pattern of male-sterility and identification of restorer lines

To determine whether the male-sterility trait discovered in this study was heritable and to examine the inheritance pattern, both the new MS plant and the Ogura MS lines were cross-pollinated with eight different male-fertile breeding lines. Male-fertility of progenies from each cross was examined. The results showed that the F_1 progenies originating from the crosses between the new MS plant and the eight male-fertile lines were all male-sterile (Table 2), implying that the male-sterility in this radish was heritable. These results also suggested that male-sterility was conditioned by cytoplasmic factors. The male-sterility of these F_1 progenies was maintained in subsequent generations by pollinating with other diverse normal breeding lines (data not shown).

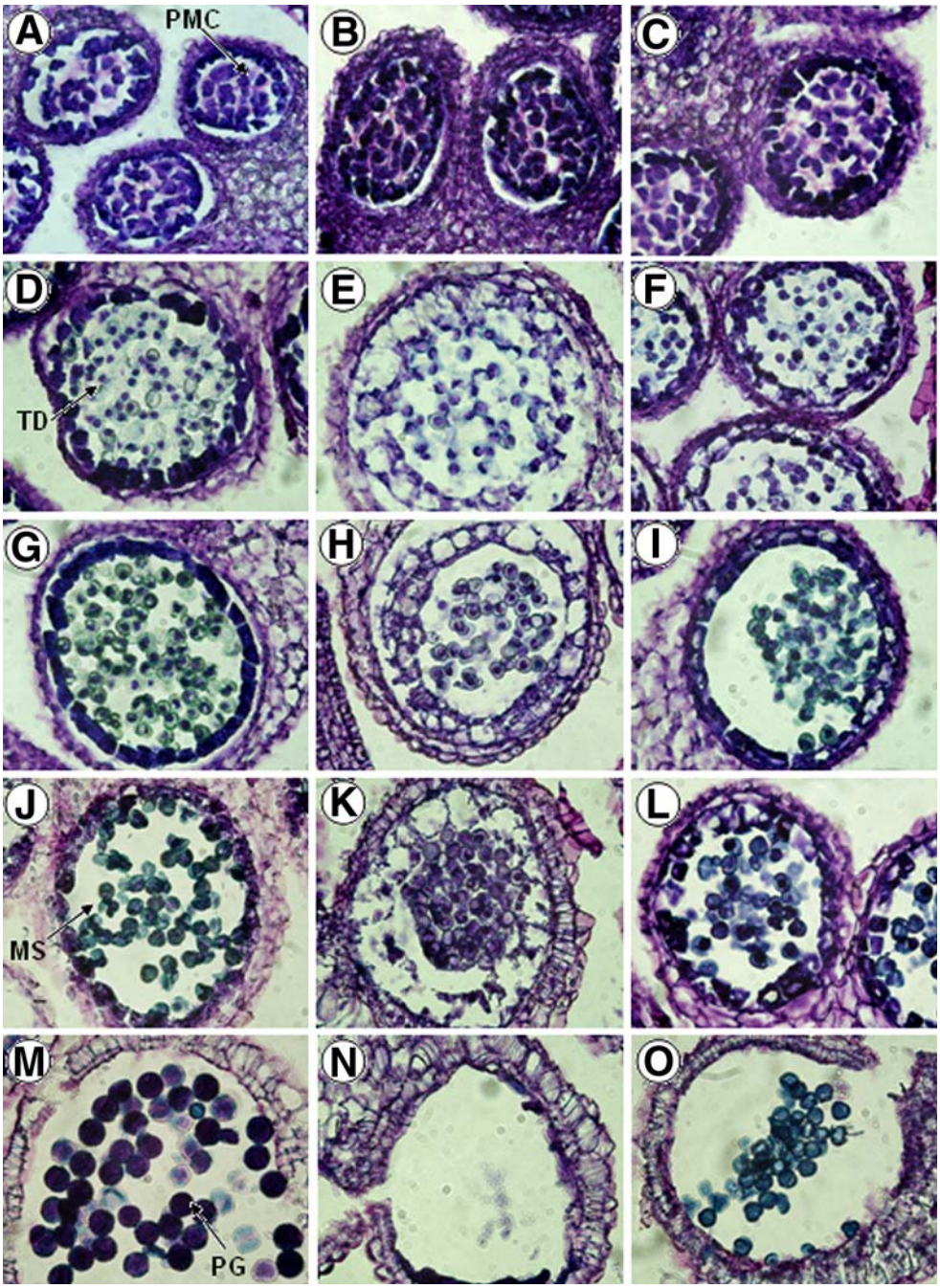

Fig. 3 Bright-field photographs of pollen development stages. Normal radish (**a**, **d**, **g**, **j**, **m**), Ogura male-sterile radish (**b**, **e**, **h**, **k**, **n**), and the newly identified male-sterile radish (**c**, **f**, **i**, **l**, **o**). The magnification factor of each stage was ×400. *PMC* pollen mother cells, *TD* tetrads, *MS* microspores, *PG* pollen grains

The progenies from the crosses between the Ogura and the same set of eight normal breeding lines showed male-sterile, male-fertile, or segregating phenotypes depending on the male parents as expected (Table 2). Ogura CMS is known as cytoplasmic-genic male-sterility in which the male-sterility-inducing factors are present in the mitochondrial genome and their restorer-of-fertility genes reside in the nuclear genomes (Hanson and Bentolila 2004).

These results also showed that restorer lines of Ogura CMS failed to restore fertility of the new male-sterility, indicating that the *orf138* gene, which is the male-sterility inducing gene in Ogura CMS (Bonhomme et al. 1991; Grelon et al. 1994), is not the male-sterility factor of this new MS plant. Considering only this result, the new male-sterility is likely to be induced by cytoplasmic factors, but there are no restorer lines, making it a complete CMS. In search

Table 2 Male-fertility of the F_1 progeny originating from crosses between male-sterile lines and diverse male-fertile breeding lines

Combinations	Number of F_1 progenies		
	Male-sterile	Male-fertile	Total
New MS × R12	20	0	20
New MS × R20	16	0	16
New MS × R29	16	0	16
New MS × R30	8	0	8
New MS × R48	12	0	12
New MS × R91	14	0	14
New MS × R111	15	0	15
New MS × R124	16	0	16
Ogu-R49 × R12	0	20	20
Ogu-R106 × R20	0	11	11
Ogu-R106 × R29	0	8	8
Ogu-R106 × R30	0	11	11
Ogu-R106 × R48	0	14	14
Ogu-R106 × R91	13	0	13
Ogu-R106 × R111	0	17	17
Ogu-R106 × R124	4	4	8

Ogu- Ogura CMS breeding lines

of restorers of this male-sterility, we surveyed more diverse germplasms. One accession (R171) collected from Russia completely restored male-sterility, and another accession (R121), whose origin was unclear, appeared to have heterozygous fertility-of-restorer genes since half of the progenies were male-sterile and the other half were fully male-fertile (Table 3). Although the inheritance pattern of restorer-of-fertility genes was not clearly resolved with these data, they do provide evidence that restorer genes exist for this new male-sterility.

Table 3 Male-fertility of the F_1 progeny from crosses between the new MS plant and diverse radish germplasm

Combinations	Number of F_1 progenies		
	Male-sterile	Male-fertile	Total
New MS × R73	20	0	20
New MS × R76	19	0	19
New MS × R104	19	0	19
New MS × R105	17	0	17
New MS × R109	17	0	17
New MS × R120	17	0	17
New MS × R121	8	9	17
New MS × R123	17	0	17
New MS × R162	11	0	11
New MS × R171	0	9	9

Amplification profile of the new MS plant revealed by radish mitotype classification markers

Previously, we reported molecular markers that amplified eight different radish mitochondrial genome fragments for classification of radish mitotypes (Kim et al. 2007). According to that report, diverse radish germplasm was successfully classified into three mitotypes (Ogura, DBRMF1, and DBRMF2). To examine whether the newly identified male-sterility belongs to one of the three previously reported mitotypes, PCR amplification profiles of the new MS lines were compared with three other mitotypes. The amplification pattern did not match any of the three mitotypes (Fig. 4), indicating that the mutant MS lines contained a new mitotype. Particularly, the marker for amplification of molecule 6, which had been reported as the most prominent difference among the three mitotypes (Kim et al. 2007), repeatedly failed to amplify any distinct PCR products. In addition, accession R171 which could restore the fertility of this male-sterility, also possessed the same new mitotype as that of the new MS line (Fig. 4). The unique PCR amplification pattern of the new MS plant was maintained in the F_1 progenies that originated from the crosses between this new MS plant and the normal breeding lines. The different amplification profile suggests that this mitotype might not arise from abrupt recent mutations. Therefore, we designated this new male-sterility and mitotype as Dongbu cytoplasmic and genic male-sterility (DCGMS).

Discussion

Discovery of a new male-sterility in radishes

Since its discovery in 1968, Ogura CMS has been extensively studied with its male-sterility inducing factor that resides in the mitochondrial genome and the restorer-of-fertility gene that resides in the nuclear genome. In addition, Ogura CMS has been exploited to develop radish F_1 hybrid cultivars. However, few efforts to search for a new source of male-sterility have been attempted in radishes. Here, we reported a new CMS radish and a mitotype. Cytological examination of the pollen development indicated that the later stages of microsporogenesis would be impaired in the new MS (DCGMS) plant compared with the Ogura CMS. Male-sterility inducing factors in the mitochondrial genome were different in DCGMS because restorer lines of Ogura CMS failed to restore DCGMS fertility. Indeed, the PCR amplification profile of mtDNA fragments implied that the DCGMS mitochondrial genome organization was significantly different from that of the Ogura CMS. In total, these results presented evidence that DCGMS was a novel radish male-sterility.

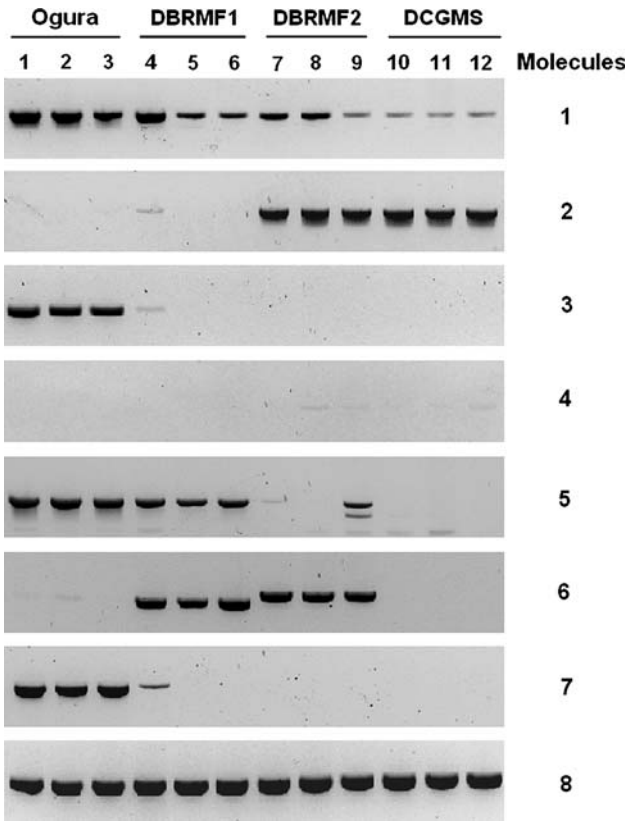

Fig. 4 PCR amplification profiles of molecular markers for radish mitotype classification. *Lanes 1–3*, *4–6*, *7–9* representative breeding lines for three mitotypes: Ogura, DBRMF1, and DBRMF2, respectively. *Lane 10* new male-sterile plant. *Lane 11* F_1 hybrid originating from the cross between the new male-sterile plants and a normal male-fertile breeding line. *Lane 12* R171, a restorer line for the new male-sterile radish. The gene organization of molecules 1–8 were depicted in Kim et al. (2007)

Nahm et al. (2005) also reported a CMS radish (NWB CMS), which was different from Ogura CMS, and a specific molecular marker for its detection. Although the phenotype of NWB CMS male-sterility appears to be similar to that of DCGMS male-sterility, it is not clear whether they are the same male-sterility. Indeed, when the molecular marker reported by Nahm et al. (2005) for exclusive detection of NWB CMS was tested with DCGMS and diverse radish germplasms, the positive bands appeared in almost all radish germplasms, though the band intensity varied depending on the radish lines (data not shown). The positive band should not have appeared in any of the radishes other than NWB CMS according to the report of Nahm et al. (2005). This discrepancy might be due to the dominant nature of this molecular marker and the complexity of the mitochondrial genomes caused by the stoichiometry of the subgenomic mtDNAs (Small et al. 1989; Bellaoui et al. 1998; Arrieta-Montiel et al. 2001). All radishes may contain the mtDNA fragment that was reported to be unique in NWB CMS radishes, though the stoichiometry of this fragment may vary among radish germplasms. Further studies are under way to compare these two new radish MS lines.

Interaction between mitochondrial MS-inducing factors and nuclear restorer genes

A novel radish mitotype responsible for the male-sterility of DCGMS lines was identified in this study. The PCR amplification profile of mtDNA fragments suggested that the mitochondrial genome organization of the new mitotype was different from the other three mitotypes: Ogura, DBRMF1, and DBRMF2 (Kim et al. 2007). Makaroff and Palmer (1988) also reported that Ogura and normal (DBRMF2) radishes had highly rearranged mitochondrial genomes. Extensive variation in the mitochondrial genome organizations observed among four different mitotypes appears to be surprising, considering if all four cytoplasms belonged to the same species. Like alloplasmic male-sterility, which frequently results from inter-specific hybridization (Leino et al. 2005; Shinada et al. 2006), highly variable mtDNA organization among four radish mitotypes might be responsible for the male-sterility of Ogura and in DCGMS lines due to the incompatibility between specific mitochondrial and nuclear genomes. Dynamic mtDNA rearrangement mediated by ubiquitous short repeat sequences often produces chimeric open reading frames, which may produce male-sterility inducing factors (Palmer 1988; Ullrich et al. 1997). The ease of acquiring promoters by co-transcription with adjacent genes may accelerate the creation of active chimeric genes. In fact, all known mitochondrial male-sterility inducers are chimeric mitochondrial genes (Hanson and Bentolila 2004).

Radish mitochondria may have experienced a rapid mitochondrial genome rearrangement after speciation, resulting in the creation of several chimeric open reading frames. The negative effect of these chimeric genes on mitochondrial functions may have been negated by nuclear genes. The PPR gene family is the best-known group of nuclear genes that can suppress the mitochondrial chimeric gene activity (Small and Peeters 2000). Unknown active mechanisms are thought to accelerate the duplication or mutation of PPR genes to cope with the abrupt appearance of chimeric mitochondrial genes (Lurin et al. 2004). The identity of the Ogura CMS restorer-of-fertility gene was found to be a PPR gene (Brown et al. 2003; Desloire et al. 2003; Koizuka et al. 2003). However, not all mitochondrial chimeric genes are suppressed by the nuclear counterparts due to inactivation by mutations or a possible lack of PPR genes. Geographical isolation of one radish mitotype in a limited area might not provide sufficient time for the nuclear genome to prepare active suppressors. The low frequency of the DCGMS mitotype and the lack of restorer lines in East Asian regions, especially in Korea and Japan,

might be caused by local isolation of the DCGMS mitotype in specific regions far from East Asia. It is worth surveying the DCGMS mitotype frequency in West Asian regions such as Uzbekistan or Russia where radish accessions containing the DCGMS mitotype were collected. Accession R171, introduced from Russia, was completely male-fertile even though it contained the DCGMS mitotype, meaning that this accession possessed the nuclear suppressor gene for the male-sterility of DCGMS lines. It is plausible that the frequency of restorer lines for DCGMS male-sterility would be high in West Asia. Large-scale examination of the distribution of diverse germplasms in the radish mitotype is currently under way in our laboratory.

Use of the newly identified MS radish to develop F_1 hybrid cultivars

Ogura CMS has contributed to the reliable production of commercial radish F_1 hybrid varieties. Male-sterility as a tool for preventing self-pollination of the female parent in F_1 hybrid production is superior to self-incompatibility in which stigma repels self-pollens, because the male-sterility system greatly increases the purity of the F_1 hybrids. However, the low frequency of maintainer lines, whose restorer-of-fertility genotype is homozygous recessive for Ogura CMS, has been an obstacle in developing diverse radish F_1 cultivars in Korea. The newly identified CMS in this study will be a valuable source of male-sterility since the frequency of the maintainer lines was extremely low in elite breeding lines commonly used in Korean radish breeding programs (Tables 2, 3). The new radish male-sterility can be introduced into *Brassica* species to develop F_1 hybrid breeding system using male-sterility as an alternative to Ogura CMS. However, additional studies on the stability of male-sterility in diverse environmental conditions are required before the new male-sterility system could be used for this purpose. Aside from its commercial use, the new male-sterility and mitotype identified in this study will be valuable for studying interactions between dynamic mitochondrial genomes and nuclear genomes. The role of the PPR gene family in such interactions is an intriguing topic for future studies.

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