Research Communication

Analysis of Chloroplast Genome of Two Cytoplasmic Male Sterile Lines Derived from Interspecific Chimera and Intergeneric Somatic Hybrid in *Brassicaceae*

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Two new types of cytoplasmic male sterile (CMS) lines have been developed, one by *in vitro* chimera formation between *Brassica rapa* and *B. oleracea* and another by protoplast fusion of *B. oleracea* with *Raphanus sativus*. These CMS lines harbored an *Ogura*-type mitochondrial gene, *orf138*. They expressed chlorosis at a young stage and their chloroplast genome changed to the type of *Ogura* radish based on restriction fragment length polymorphism (RFLP) analysis. Thus, we attempted to study the relationships among cytoplasmic male sterility, chlorosis and the variation of the chloroplast genome using the *matK* and RuBisCO genes. The two CMS lines, normal and male sterile radishes exhibited similar *matK* gene sequences, while *B. rapa* and *B. oleracea* showed *matK* sequences of the radish type in very low copy numbers. The CMS lines displayed an identical RFLP pattern with that of the male sterile radish in the RuBisCO gene region. These phenomena indicated that the chloroplast genome organization had changed from *Brassica* to *Raphanus* type or *Raphanus* CMS type by CMS induction, and suggested the existence of heteroplasia in the *Brassica* chloroplast.

Key Words: Cytoplasmic male sterility, interspecific *Brassica* chimera, chloroplast, *matK*.

Introduction

We developed two new cytoplasmic male sterility (CMS) lines, one derived from the interspecific *Brassica* chimera between ‘Komatsuna’ (*B. rapa*) and red cabbage, ‘Ruby Ball’ (*B. oleracea*) (Noguchi *et al.* 1992, Noguchi and Hirata 1994, Motegi *et al.* 2003), and another from the intergeneric somatic hybrid between ‘Shogoin’ (*R. sativus*) and ‘Ruby Ball’ (Kameya *et al.* 1989, Motegi *et al.* 2003). Molecular analysis of the mitochondrial genome was performed to elucidate the induction mechanism of cytoplasmic male sterility (male sterility, hereafter) (Motegi *et al.* 2003). These CMS lines harbored the *Ogura*-specific *orf138* gene, responsible for radish CMS (Makaroff *et al.* 1991, Yamagishi and Terachi 2001). The CMS lines were very stable without anthers or pollen, i.e., fertility was not recovered under fluctuating conditions.

These two new types of CMS lines are suitable plant materials for studying the CMS regulation system. Southern blot analysis of the chimera-derived CMS line using 16 mitochondrial probes revealed band patterns similar to that of the ‘Kosena’ radish mitochondrial genome (Hirata *et al.* 2000). In addition, the somatic hybrid between normal fer-

tile radish and normal fertile cabbage clearly maintained a genome with a small amount of *Ogura* type cytoplasm (Kameya *et al.* 1989, Motegi 1999).

Previously, we analyzed these CMS lines using the mitochondrial *Ogura*-specific *atp6* (Makaroff *et al.* 1989) and *orf138* genes (Makaroff *et al.* 1991). The chimera-derived CMS, protoplast fusion-derived CMS and radish with *Ogura* CMS displayed a similar genome structure (Hirata *et al.* 2000, Motegi *et al.* 2003). Interestingly, these two new CMS types showed similar RFLP patterns in the mitochondria and chloroplast, although their origin was different. Furthermore, these CMS lines expressed chlorosis at the seedling stage. It is generally recognized that *Brassica* CMS lines express chlorosis (Ohkawa 1986) and that the chloroplast genome participates in the expression of chlorosis, while the interrelationship between the CMS and chloroplast has not been fully elucidated or was ruled out (Ohkawa 1986). However, because of the similarity of the changes in the mitochondrial and chloroplast RFLP patterns to those of the *Ogura* CMS type, we analyzed the organization of the chloroplast genome compared with that of the mitochondrial genome (Motegi *et al.* 2003). Motegi (1999) performed a Southern blot analysis using *Bam*HI-digested BAC clones from the rice chloroplast genome, because the BAC clones were constructed in rice at that time (Hirai *et al.* 1985). As a result, differences in the RFLP patterns between fertile radish and a CMS radish were revealed.

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In order to gain a better understanding of the structure of the chloroplast genome in the CMS lines, we performed an analysis of the chloroplast genome in the region that harbored the *matK* and RuBisCO genes. The *matK* gene in the chloroplast codes for the intron within the *trnK* gene (Neuhaus and Link 1987, Liere and Link 1995, Ostheimer *et al.* 2003). Classification and systematics analyses have been widely performed at the generic and specific levels using the specific *matK* sequence polymorphism (Steele and Vilgalys 1994, Soltis *et al.* 1996, Xiang *et al.* 1998, Wu *et al.* 2000, Koch *et al.* 2001). Both of the CMS lines displayed a structure similar to that of radish in *matK* gene region, while, they exhibited a RFLP pattern specific to that of the male sterile radish of the RuBisCO gene site.

Materials and Methods

Plant materials

Our laboratory has been maintaining cytoplasmic male sterile (CMS) lines originating from chimeras obtained by interspecific *in vitro* grafting between 'Komatsuna' (*Brassica rapa*) and 'Ruby Ball' (*B. oleracea*) (Noguchi *et al.* 1992). These CMS lines were designated as chimera-derived-CMS (CDC) lines. On the other hand, the CMS cabbage, an intergeneric somatic hybrid, was synthesized by protoplast fusion between *B. oleracea* cv. 'Ruby Ball' and *Raphanus sativus* cv. 'Shogoin' by Kameya *et al.* (1989). These CMS lines were designated as protoplast-fusion-derived CMS (PDC) lines. We used these two types of CMS lines and respective parental plants in the present study (Hirata *et al.* 2000, Motegi *et al.* 2003). As a comparable standard, the *Ogura*-type CMS cv. 'MS-Gensuke' (*R. sativus*) was adopted (Ogura 1968, Makaroff *et al.* 1991, Yamagishi and Terachi 2001). Normal fertile radish 'Uchiki-Gensuke' was used as a maintainer for 'MS-Gensuke', while 'Comet' (*R. sativus*) as a restorer of *Ogura* CMS. The *B. rapa* Noko (N) line was also used for the RFLP analysis.

DNA extraction and primer design

Total DNA was extracted from young leaves by the modified CTAB method (Aldrich and Cullis 1993). *MatK* was amplified by the polymerase chain reaction (PCR) method with *matK* gene-specific sequences as primers. The primers were designed from the complete chloroplast sequences based on the clustal W analysis in *Oryza sativa* (NC 001320), *Zea mays* (NC 001666), *Arabidopsis thaliana* (NC 001879), *Nicotiana tabacum* (NC 000932) and *Spinacia oleracea* (NC 002202) chloroplasts.

PCR amplification and TA cloning

PCR amplification was performed in a 25 µl reaction solution containing 16.87 µl of sterile water, 2.5 µl of 10 × *Taq* polymerase reaction buffer (Takara Bio Co. Ltd. Otsu, Shiga, Japan), 2.0 µl of 2.5 mM dNTPs in an equimolar ratio, 0.13 µl of *Taq* DNA polymerase (5 unit/µl) (Takara Bio Co. Ltd.), 1.0 µl of 12.5 µM *matK* gene-specific cpp02 (5'-CGC

GTC TCT CTA AAA TTG CAG-3') and cpp03 (5'-ATC WAT TCA TTC AAY ATT TCC-3') primers (Fig. 1), 1.5 µl of 25 mM MgCl₂ and 1.0 µl of template DNA (25 ng/µl). The solutions were first denatured for 2 min at 94°C, followed by 30 cycles of amplification consisting of 30 sec at 94°C (denaturation), 30 sec at 50°C (annealing), and 2 min at 72°C (polymerization). After amplification, the products were ligated into pT7blue vectors (Novagen Co. Madison, WI, USA) and used for DNA sequencing analysis.

The purified plasmid DNA of each clone was sequenced on the ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster, CA, USA) using the DYEnamic ET terminator cycle sequencing kit (Amersham Biosciences, Piscataway, NJ, USA). DNA sequences were aligned manually by using GENETIX-WIN 4.0 (GENETYX Co. Tokyo, Japan). The BLAST and the CLUSTAL W programs were used for searching the homology among nucleotide sequences. PCR primer was designed from a sequence specific to 'CDC', 'PDC', 'Shogoin' and 'MS-Gensuke' *matK* gene, and was designated as Radish sp (5'-ATC TTA GTC TTA CTG CTT ACT G-3'). PCR was conducted with the primer pair of Radish sp and cpp03 (Fig. 1). PCR was performed by the above method with 30 and/or 45 cycles.

Furthermore, a new specific marker that can amplify the 8kb region present far from the *matK* gene region was designed to investigate RFLP between the CMS and the fertile one. The region included the RuBisCO gene part on the data base search (abbreviated as RuBisCO gene sequence). PCR primers were designed from a conserved sequence in 'CDC', 'PDC' and 'MS-Gensuke'. The forward (5'-GGA ATC TTC TAC TGG TAC ATG GAC AAC TGT-3') and the reverse (5'-GGC TAA ATG GCA ATT GGC ACA TAC AAT-3') primers were used for PCR amplification. PCR was performed using KOD plus DNA polymerase (Toyobo Co., Ltd. Osaka, Osaka, Japan). The solutions were firstly denatured for 2 min at 94°C, followed by 30 cycles of amplification consisting of 20 sec at 94°C (denaturation), 8 min at 68°C (annealing of primers and polymerization). The PCR product was used to synthesize the probe for CDC amplification.

Southern blot analysis

Southern blot analysis was performed by a conventional method (Roche Diagnostics, Mannheim, Germany; Tateishi *et al.* 2001, 2002). Genomic DNA (10 µg) was digested with the *MspI* restriction enzyme. Digested DNAs were separated by electrophoresis on 0.8% TAE-agarose gel and transferred onto a Hybond N+ membrane (Amersham Biosciences). The DIG-labeled *matK* gene PCR product and RuBisCO gene PCR product were used as hybridization probes. The membrane was washed once at 68°C for 15 min after hybridization.

Results and Discussion

We amplified the *matK* gene from the CMS lines and

fertile parental lines using the cpp02 and cpp03 primers (Fig. 1). Based on the comparison of the sequencing data, the PCR products were found to show almost the same size as follows : 1,765 bp in ‘CDC’, 1,762 bp in ‘PDC’, 1,758 bp in ‘Ruby Ball’, 1,756 bp in ‘Komatsuna’, 1,765 bp in ‘Shogoin’ and 1,764 bp in ‘MS-Gensuke’. These sequences corresponded to those of the *matK* genes based on BLAST complementation (Koch *et al.* 2001). ‘CDC’ and ‘PDC’, the CMS radish and fertile ‘Shogoin’ displayed a common seven-base pair region that was not found in ‘Ruby Ball’ and ‘Komatsuna’ (Fig. 2).

Using the specific primers (Radish sp and cpp03), the band was amplified by PCR with 30 cycles in the CMS lines and fertile radish, but not in ‘Ruby Ball’ or ‘Komatsuna’. However, ‘Ruby Ball’ and ‘Komatsuna’ showed a similar molecular size of band by PCR with 45 cycles (Fig. 3). These amplified bands obtained with 45 cycles were cloned into the pT7 blue vector and sequenced, showing almost the same size, namely 1,271 bp in ‘CDC’, ‘PDC’, ‘Ruby Ball’, and ‘Komatsuna’, as well as 1,270 bp in ‘Shogoin’ and 1,272 bp in ‘MS-Gensuke’ (Fig. 2 and Fig. 3). In addition, the *MspI* restriction site was also found at a Radish-specific position in the sequence. The fertile line had two *MspI* restriction sites and the CMS line had three sites in the *matK* sequence (Fig. 1, Fig. 2 and Fig. 3).

Although no PCR products were obtained in the fertile *Brassica* plants with the Radish sp primer after the conventional 30 cycles, a faint band was observed with 45 cycles, suggesting the existence of heteroplasmic molecules like in the case of mitochondrial *orf138*. Our findings indicated the presence of two different molecular species with different copy numbers. This heteroplasmic phenomenon has been well documented in the case of the mitochondrial *orf138* and related genes (Makaroff *et al.* 1991). In the previous experiment, three molecules were observed and the copy numbers varied (1,000 times difference) between the CMS lines and

Fig. 1. Primer positions in the *matK* region for identification of the polymorphism in ‘CDC’ and *Arabidopsis thaliana*. *MatK* gene regions of ‘CDC’ and *Arabidopsis thaliana* are comparatively shown. Designed primers cpp02, cpp03 and Radish sp are indicated by arrows. ① corresponds to the site lacking seven bases.

CDC	1199	AATTATTTTGTATTATCAAAAGTCATTTATTTCTATTATTAATGTTTCATACAGTAGCA	1258
PDC	1199	AATTATTTTGTATTATCAAAAGTCATTTATTTCTATTATTAATGTTTCATACAGTAGCA	1258
Shogoin	1199	AATTATTTTGTATTATCAAAAGTCATTTATTTCTATTATTAATGTTTCATACAGTAGCA	1258
MS Gensuke	1198	AATTATTTTGTATTATCAAAAGTCATTTATTTCTATTATTAATGTTTCATACAGTAGCA	1257
Ruby ball	1199	AATTATTTTGTATTATCAAAAGTCATTTATTTCTATTATTAATGTTTCATACAGTAGCA	1251
Komatsuna	1199	AATTATTTTGTATTATCAAAAGTCATTTATTTCTATTATTAATGTTTCATACAGTAGCA	1251
CDC SP	1199	AATTATTTTGTATTATCAAAAGTCATTTATTTCTATTATTAATGTTTCATACAGTAGCA	1258
PDC SP	1200	AATTATTTTGTATTATCAAAAGTCATTTATTTCTATTATTAATGTTTCATACAGTAGCA	1259
Shogoin SP	1199	AATTATTTTGTATTATCAAAAGTCATTTATTTCTATTATTAATGTTTCATACAGTAGCA	1258
MS Gensuke SP	1200	AATTATTTTGTATTATCAAAAGTCATTTATTTCTATTATTAATGTTTCATACAGTAGCA	1259
Ruby ball SP	1199	AATTATTTTGTATTATCAAAAGTCATTTATTTCTATTATTAATGTTTCATACAGTAGCA	1258
Komatsuna SP	1199	AATTATTTTGTATTATCAAAAGTCATTTATTTCTATTATTAATGTTTCATACAGTAGCA	1258
CDC	1259	STAGACTAGATAAATTTACTGAGTATTGAATTCGCAGTCCTATTTATAGGAACCA	1318
PDC	1259	STAGACTAGATAAATTTACTGAGTATTGAATTCGCAGTCCTATTTATAGGAACCA	1318
Shogoin	1259	STAGACTAGATAAATTTACTGAGTATTGAATTCGCAGTCCTATTTATAGGAACCA	1318
MS Gensuke	1258	STAGACTAGATAAATTTACTGAGTATTGAATTCGCAGTCCTATTTATAGGAACCA	1317
Ruby ball	1252	STAGACTAGATAAATTTACTGAGTATTGAATTCGCAGTCCTATTTATAGGAACCA	1311
Komatsuna	1252	STAGACTAGATAAATTTACTGAGTATTGAATTCGCAGTCCTATTTATAGGAACCA	1311
CDC SP	1259	STAGACTAGAT-----	1271
PDC SP	1260	STAGACTAGAT-----	1272
Shogoin SP	1259	STAGACTAGAT-----	1271
MS Gensuke SP	1260	STAGACTAGAT-----	1272
Ruby ball SP	1259	STAGACTAGAT-----	1271
Komatsuna SP	1259	STAGACTAGAT-----	1271

Fig. 2. Comparison of *matK* sequences between CMS lines and fertile lines. ‘CDC’ sp, ‘PDC’ sp, ‘Ruby Ball’ sp, ‘Komatsuna’ sp, ‘Shogoin’ sp and ‘MS-Gensuke’ sp denote the radish specific sequence, respectively. The Radish sp primer site is underlined.

Fig. 3. PCR analysis using the Radish sp primer and cpp03 primer with 30 and 45 cycles. ‘CDC’, ‘PDC’, C and M denote chimera-derived CMS, protoplast fusion-derived CMS, restorer radish cv. ‘Comet’, and molecular size marker (2.0 kbp).

fertile mitochondria (Motegi *et al.* 2003).

The RFLP pattern of the CMS lines in the *matK* region was similar to that of fertile radish and different from that of fertile *B. rapa* and *B. oleracea* (Fig. 4). However, bands with a similar size to that of 'CDC', 'PDC', 'Shogoin' and 'MS-Gensuke' were also observed in 'Komatsuna' and 'Ruby Ball', though the latter two varieties showed very faint bands. On the other hand, 'PDC', which is the asymmetric somatic hybrid between 'Ruby Ball' and 'Shogoin', exhibited molecules specific to both parents. We found that the *matK* gene in 'CDC' had a homologous sequence to that of radish. Interestingly, the results suggested that in fertile *Brassica*, the radish type *matK* gene was present in very low copy numbers. The causal mechanism of the shift of the *matK* gene structure of the CMS lines to the radish type has not been determined and further studies should be carried out (Fig. 4 and Fig. 5).

The results obtained by Southern blot analysis using the RuBisCO probe were different from those of the RFLP analysis in the *matK* gene region (Fig. 5). Amplification bands with the RuBisCO gene primer could identify differences among fertile *Brassica* and the radish parents, the CMS lines and CMS radish. Differences in the RFLP pattern between the fertile parents and CMS lines ('CDC', 'PDC' and 'MS-Gensuke') were observed. The pattern of the CMS lines was different from that of the radish variety, 'Shogoin' with normal cytoplasm. The results indicated that the CMS lines showed a different RuBisCO structure from that of the fertile lines, suggesting some relation with the expression of chlorosis in the CMS lines.

The results of this experiment suggested that a stoichiometric shift might have occurred in the chloroplast genome of the two CMS lines by a still unknown mechanism. The analysis of the cytoplasmic gene structure in more detail might shed light on the organelle genome structure and the CMS regulation mechanism(s) of the two lines.

Fig. 4. Southern blot analysis of CMS lines and fertile plants probed with the PCR product of the *matK* gene in CDC line. Genomic DNA of each material was digested with *MspI*. The arrow indicates the minor bands. 1, 'CDC'; 2, 'PDC'; 3, 'Ruby Ball'; 4, 'Komatsuna'; 5, 'Shogoin'; 6, 'MS-Gensuke'; 7, 'N line'; 8, 'Comet'.

Fig. 5. Southern blot analysis of CMS lines and fertile plants probed with the PCR product of the RuBisCO gene in the CDC line. Genomic DNA of each material was digested with *MspI*. 1, 'CDC'; 2, 'PDC'; 3, 'Ruby Ball'; 4, 'Komatsuna'; 5, 'Shogoin'; 6, 'MS-Gensuke'; 7, 'N line'; 8, 'Comet'.

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