The mitochondrial genome of an asymmetrically cell-fused rapeseed, *Brassica napus*, containing a radish-derived cytoplasmic male sterility-associated gene

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Cytoplasmic male sterility (CMS) is an agronomically important trait whose causative genes are located in the mitochondrial genome. A CMS rapeseed, *Brassica napus* ‘SW18’, was made 25 years ago by an asymmetric (or “donor-recipient”) cell fusion between *B. napus* ‘Westar’ and a CMS radish (*Raphanus sativus* ‘Kosena’), in order to transfer the radish CMS-associated gene without disturbing the rapeseed features. Here, we determined the nucleotide sequences of the mitochondrial genomes of Kosena radish and SW18. SW18 has a recombinant mitochondrial genome, which includes the whole 222-kb genome of Westar (54 genes) and a total of 23 kb insertions of four fragments from Kosena radish (three genes: *orf125*, *trnM* and *atp1*). All of the Kosena radish-derived fragments in the SW18 mitochondrial genome had sequences at their ends (ranging from 63 bp to 628 bp) that are identical to the sequences at the sites of insertion on the Westar rapeseed-derived mitochondrial genome. This suggests that these insertions were mediated by homologous recombination. These results confirm at the nucleotide level that a desired CMS-associated gene (*orf125*) along with a few extraneous genes from radish were successfully transferred.

**Key words:** asymmetric cell fusion, donor-recipient cell fusion, plant mitochondrial genome

**INTRODUCTION**

Cytoplasmic male sterility (CMS) is frequently used in F1 hybrid breeding for efficient F1 seed production, because it causes bisexual plants to become female, resulting in secure outcrossing (Chen and Liu, 2014). Many *Brassica* vegetables show strong hybrid vigor, but good CMS is scarce. Therefore, Ogura-type CMS in the radish, *Raphanus sativus* L. (Brassicaceae) (Ogura, 1968), is now widely transferred to *Brassica* vegetables (Yamagishi and Bhat, 2014). Early attempts to transfer Ogura cytoplasm to *Brassica* vegetables by hybridization between radish and rapeseed and backcrossing to the rapeseed failed to produce a good product, because genes other than CMS genes that were acquired from radish mitochondrial or plastid genomes caused inappropriate phenotypes (yellowish and slow-growth) (Pelletier et al., 1983; Yamagishi and Bhat, 2014). Cell fusion is another strategy for introducing genes from distinct species. Plants regenerated from fused cells and their progeny are known to have only one of the parental plastid genomes while the mitochondrial genome is recombined from the parental ones (Belliard et al., 1979; Pelletier et al., 1983; Wang et al., 2012; Sanchez-Puerta et al., 2015). Sakai and Imamura (1992) tried to produce a CMS rapeseed harboring the CMS-associated gene *orf125* from *R. sativus* ‘Kosena’, by using asymmetric cell fusion (donor-recipient cell fusion) between *Brassica napus* ‘Westar’ and an X-ray-irradiated Kosena radish (Fig. 1) (Sakai and Imamura, 1992). The resultant line, *B. napus* ‘SW18’, having 38 nuclear chromosomes (the same as rapeseed, 2n = 38, but not radish, 2n = 18) was consecutively backcrossed (more than six times) and showed growth phenotypes identical to those of Westar rapeseed except for CMS (Sakai and Imamura, 1992; Iwabuchi et al., 1999).

Here, we compared the mitochondrial genome of SW18 rapeseed, the asymmetrically cell-fused line, with the mitochondrial genomes of the two parent lines, Westar...
rapeseed (Handa, 2003) and Kosena radish, the CMS donor line, to see which genes (and which DNA fragments) were transferred to, or recombined in, the SW18 mitochondrial genome.

MATERIALS AND METHODS

Plant materials and growth conditions Seeds (B. napus ‘Westar’ and ‘SW18’, and R. sativus ‘Kosena’) were obtained from Dr. Jun Imamura (Sakai and Imamura, 1992). These plants were grown on Jiffy pellets (Jiffy Products) at 22 °C under 16 h of diurnal light or continuous light (100 μmol m⁻² sec⁻¹). Total DNA was isolated by Plant DNeasy Mini Kit (Qiagen) from leaves of 14-day-old plants.

DNA sequencing, assembly and analyses Paired-end (350-bp) libraries of genomic DNAs were constructed and sequenced on Illumina HiSeq 2500 and HiSeq10X platforms by BGI. The Kosena radish sequencing generated 97,692,126 reads with a total length of 14,751,511,026 bp, while the SW18 rapeseed sequencing generated 97,191,262 reads with a total length of 14,675,880,562 bp. The reads were mapped to the reference sequences of R. sativus ‘MS-Gensuke’ (NC_018551) and Westar rapeseed (NC_008285), respectively. Reads mapping to the reference sequences of the mitochondrial genomes (530,362 reads of Kosena and 455,699 reads of SW18) were collected and re-used for generating de novo contigs with the software Geneious (Biomatters). Reads of the nuclear and chloroplast genomes that were erroneously mapped to the mitochondrial genomes could be easily detected by differences in the depths of their read coverage (about ten times less or ten times more, respectively, than those of the genuine reads for the mitochondrial genomes) and they were manually removed or ignored. The generated de novo contigs were manually connected by the sequences at their ends and the connections were confirmed by long PCRs. The sequences determined in this study were deposited in DDBJ as accession numbers AP018472 (Kosena radish mitochondrial genome), AP018473, AP018474 and AP018475 (sub-genome 1, 2 and 3, respectively, of the SW18 rapeseed mitochondrial genome).

PCR analyses Long PCR experiments to confirm the mitochondrial genome structures mapped by Illumina short reads were done using Prime Star GXL DNA polymerase (TaKaRa) according to the manufacturer’s instruction.

RESULTS & DISCUSSION

Kosena radish mitochondrial genome The mitochondrial genome of Westar rapeseed, the recipient line of asymmetric cell fusion, has a length of 221,853 bp, 34 protein-coding genes, three rRNAs and 17 tRNAs (Handa, 2003), while that of the donor line, Kosena radish, has
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a length of 258,424 bp, 34 protein-coding genes, three rRNAs and 18 tRNAs (Fig. 2 and Table 1). The Kosena mitochondrial genome displays 99.959% identity with the previously sequenced mitochondrial genome of an Ogura-type CMS radish, MS-Gensuke (258,426 bp, 34 protein-coding genes, three rRNAs and 18 tRNAs, Tanaka et al., 2012). The two radish mitochondrial genomes have the same order of genes as each other except for a single 90-kb inversion. The inverted region has a pair of 442-bp inverted repeats at its ends (100% identity between the inverted repeat sequences), which are the second-largest pair of repeats in the genome (the largest repeat is 9,731 bp). Park et al. (2013) reported that an Ogura-type CMS radish has multiple master-circle genome structures with a difference of a 79,976-bp inversion with a pair of 311-bp inverted repeats at the ends; however, this inversion is distinct from that reported here.

Mitochondrial genome of the asymmetrically cell-fused SW18 rapeseed The mitochondrial genome of SW18 appears to consist of three circular subgenomes (Fig. 3A, 227,181 bp, 156,632 bp and 14,497 bp). They have long identical sequences (region W2-C, 4,136 bp) between them, so that they can be drawn as a master circle with a total length of 398,310 bp (Fig. 3B). The nucleotide sequence and the structure of the first circular

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Fig. 2. Master circle of the mitochondrial genome sequence of *Raphanus sativus* ‘Kosena’. Light blue arrows show genes (or their exons) encoding 34 function-predictable proteins and 35 functional-unpredictable ORFs longer than 100 amino acids. Red arrows and triangles show 18 tRNAs and three ribosomal RNAs. The green block shows the inverted sequence compared to the mitochondrial genome of MS-Gensuke radish (AB694744), with the 442-bp inverted repeats (labeled in gray color) at the ends. Four orange arrows labeled “Region K1” to “Region K4” are the sequences found in the SW18 rapeseed mitochondrial genome (see also Fig. 3). The Kosena CMS-associated gene, *orf125*, is located on Region K2. The longest and second-longest repeat pairs (9,731 bp and 442 bp) are highlighted in gray.
Table 1. Genes in the three mitochondrial genomes

| Genes                        | Kosena radish | Westar rapeseed | SW18 rapeseed |
|------------------------------|---------------|-----------------|---------------|
| nad1 gene                    | nad1 gene     | nad1 gene       | nad1 gene     |
| nad2 gene                    | nad2 gene     | nad2 gene       | nad2 gene     |
| nad3 gene                    | nad3 gene     | nad3 gene       | nad3 gene     |
| nad4 gene                    | nad4 gene     | nad4 gene       | nad4 gene     |
| nad4L gene                   | nad4L gene    | nad4L gene      | nad4L gene    |
| nad5 gene                    | nad5 gene     | nad5 gene       | nad5 gene     |
| nad6 gene                    | nad6 gene     | nad6 gene       | nad6 gene     |
| nad7 gene                    | nad7 gene     | nad7 gene       | nad7 gene     |
| nad9 gene                    | nad9 gene     | nad9 gene       | nad9 gene     |
| cob gene                     | cob gene      | cob gene        | cob gene      |
| cob gene                     | cob gene      | cob gene        | cob gene      |
| cob gene                     | cob gene      | cob gene        | cob gene      |
| cox1 gene                    | cox1 gene     | cox1 gene       | cox1 gene     |
| cox2 gene                    | cox2 gene     | cox2 gene       | cox2 gene     |
| cox2-2 gene*                | cox2-2 gene*  | cox2-2 gene*    | cox2-2 gene*  |
| cox3 gene                    | cox3 gene     | cox3 gene       | cox3 gene     |
| atp1 gene                    | atp1 gene     | atp1 gene       | atp1 gene     |
| atp4 (orf25)                 | atp4 (orf25)  | atp4 (orf25)    | atp4 (orf25)  |
| atp6 gene                    | atp6 gene     | atp6 gene       | atp6 gene     |
| atp8 (orfB)                  | atp8 (orfB)   | atp8 (orfB)     | atp8 (orfB)   |
| atp9 gene                    | atp9 gene     | atp9 gene       | atp9 gene     |
| atp9-2 gene*                | atp9-2 gene*  | atp9-2 gene*    | atp9-2 gene*  |
| ccmB gene                    | ccmB gene     | ccmB gene       | ccmB gene     |
| ccmC gene                    | ccmC gene     | ccmC gene       | ccmC gene     |
| ccmFC gene                   | ccmFC gene    | ccmFC gene      | ccmFC gene    |
| ccmFN1 gene                  | ccmFN1 gene   | ccmFN1 gene     | ccmFN1 gene   |
| ccmFN2 gene                  | ccmFN2 gene   | ccmFN2 gene     | ccmFN2 gene   |
| rpl16 gene                   | rpl16 gene    | rpl16 gene      | rpl16 gene    |
| rpl2 gene                    | rpl2 gene     | rpl2 gene       | rpl2 gene     |
| rpl5 gene                    | rpl5 gene     | rpl5 gene       | rpl5 gene     |
| rps12 gene                   | rps12 gene    | rps12 gene      | rps12 gene    |
| rps14 gene                   | rps14 gene    | rps14 gene      | rps14 gene    |
| rps3 gene                    | rps3 gene     | rps3 gene       | rps3 gene     |
| rps4 gene                    | rps4 gene     | rps4 gene       | rps4 gene     |
| rps7 gene                    | rps7 gene     | rps7 gene       | rps7 gene     |
| orf222                       | orf222        | orf222          | orf222        |
| orf125 Kosena                | orf125 Kosena | orf125 Kosena   | orf125 Kosena |
| mttrB (tatC orfX)            | mttrB (tatC orfX) | mttrB (tatC orfX) | mttrB (tatC orfX) |
| mtrR gene                    | mtrR gene     | mtrR gene       | mtrR gene     |
| 18S rRNA                     | 18S rRNA      | 18S rRNA        | 18S rRNA      |
| 26S rRNA                     | 26S rRNA      | 26S rRNA        | 26S rRNA      |
| SS rRNA                      | SS rRNA       | SS rRNA         | SS rRNA       |
| trnC tRNA                    | trnC tRNA     | trnC tRNA       | trnC tRNA     |
| trnD tRNA                    | trnD tRNA     | trnD tRNA       | trnD tRNA     |
| trnE tRNA                    | trnE tRNA     | trnE tRNA       | trnE tRNA     |
| trnF tRNA                    | trnF tRNA     | trnF tRNA       | trnF tRNA     |
| trnM tRNA                    | trnM tRNA     | trnM tRNA       | trnM tRNA     |
| trnM tRNA**                  | trnM tRNA**   | trnM tRNA**     | trnM tRNA**   |
| trnG tRNA                    | trnG tRNA     | trnG tRNA       | trnG tRNA     |
| trnH tRNA                    | trnH tRNA     | trnH tRNA       | trnH tRNA     |
| trnI tRNA                    | trnI tRNA     | trnI tRNA       | trnI tRNA     |
| trnK tRNA                    | trnK tRNA     | trnK tRNA       | trnK tRNA     |
| trnM tRNA                    | trnM tRNA     | trnM tRNA       | trnM tRNA     |
| trnN tRNA                    | trnN tRNA     | trnN tRNA       | trnN tRNA     |
| trnP tRNA                    | trnP tRNA     | trnP tRNA       | trnP tRNA     |
| trnQ tRNA                    | trnQ tRNA     | trnQ tRNA       | trnQ tRNA     |
| trnS tRNA                    | trnS tRNA     | trnS tRNA       | trnS tRNA     |
| trnS tRNA                    | trnS tRNA     | trnS tRNA       | trnS tRNA     |
| trnS tRNA                    | trnS tRNA     | trnS tRNA       | trnS tRNA     |
| trnT tRNA                    | trnT tRNA     | trnT tRNA       | trnT tRNA     |
| trnW tRNA                    | trnW tRNA     | trnW tRNA       | trnW tRNA     |
| trnY tRNA                    | trnY tRNA     | trnY tRNA       | trnY tRNA     |
| 17 tRNAs + an additional trnM |

*atp9-2 in Kosena radish and cox2-2 in Westar rapeseed are additional copies of atp9 and cox2 in repeats.

**additional trnMs are proximal to the orf125 in Kosena and SW18.
Fig. 3. Mitochondrial genome of SW18 rapeseed. (A) Linearized sequences of the mitochondrial genome of Westar rapeseed (NC_008285, Handa, 2003), and three circularly mapped subgenomes of SW18 rapeseed (this study). Blue arrows are the common regions between Westar and SW18 rapeseeds. Orange arrows labeled as Regions K1 to K4 are identical to the regions drawn in the Kosena mitochondrial genome (see also Fig. 2). (B) A possible master circle of the SW18 mitochondrial genome recombined from the three subgenomes shown in (A). (1), (2) and (3) refer to the regions from subgenomes 1 to 3. Subgenome 3, which is inserted into subgenome 1 in this map, could be inserted into subgenome 2 as another configuration.
subgenome are similar to those of the master circular mitochondrial genome sequence of Westar rapeseed (Handa, 2003) but with an 11,612-bp inversion of the rapeseed sequence (Region W3) and a 6,211-bp insertion from the Kosena radish mitochondrial genome (Region K1) (Fig. 3A). The inversion has a pair of 147-bp inverted repeats at its ends. Moreover, the Kosena inserted sequence, Region K1, has 281-bp and 628-bp sequences at its ends that are common to the sequences of the connected ends of Regions W1 and W2, respectively. These common sequences suggest that the inversion and insertion were generated via homologous recombination events.

The second circular subgenome (subgenome 2; 156,632 bp) has the same sequence and structure as subgenome 1, except that it has a 5,651-bp Kosena-derived insertion (Region K2) instead of the middle of Region W4, 75,675 bp. In addition, the connected ends of Regions W4-A and K2 and those of Regions K2 and W4-B have common sequences (462 bp and 63 bp) (Fig. 3B). The third small 14,497-bp circular subgenome consists of two Kosena fragments of 8,250 bp and 2,755 bp (Regions K3 and K4), and another 4,136-bp Westar mitochondrial DNA sequence (Region W2-C). These connected sites also have three common sequences: 139 bp (Regions W2-C and K3), 23 bp (Regions K3 and K4) and 482 bp (Regions K4 and W2-C). All of the new connected sequences found in the SW18 mitochondrial genome have common identical sequences at both of the connected ends, suggesting that homologous recombination events were responsible for making the new genome. These three subgenomes have similar mean coverages of Illumina short reads (553, 390 and 322), suggesting that the stoichiometry of these three molecules is also similar. A comparison of the genomes (Table 1) shows that SW18 has the full set of 54 Westar rapeseed genes and only three Kosena radish mitochondrial genes, namely orf125 (the CMS-associated gene), atp1 and trnFM.

Mitochondrial genomes of plants derived from normal cell fusion typically have more than 20 recombinations with almost equal amounts of parental genome fragments (Belliard et al., 1979; Wang et al., 2012; Sanchez-Puerta et al., 2015). The SW18 mitochondrial genome has the whole circular genome of Westar with about 60% redundancy, and four partial fragments of Kosena radish (a total of 23 kb, accounting for 8.8% of the genome). Such asymmetric contributions seem to be consistent with the achievement of the previous trial (Sakai and Imamura, 1992) to transfer a CMS-associated gene to another species without greatly disturbing other phenotypes. Because methods for stably transforming plant mitochondrial genomes are currently unavailable, asymmetric cell fusion, a relatively old technique, is still a valuable method to modify the mitochondrial genome.

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REFERENCES

Belliard, G., Vedel, F., and Pelletier, G. (1979) Mitochondrial recombination in cytoplasmic hybrids of Nicotiana tabacum by protoplast fusion. Nature 281, 401–403.

Chen, L., and Liu, Y. G. (2014) Male sterility and fertility restoration in crops. Annu. Rev. Plant Biol. 65, 579–606.

Handa, H. (2003) The complete nucleotide sequence and RNA editing content of the mitochondrial genome of rapeseed (Brassica napus L.): comparative analysis of the mitochondrial genomes of rapeseed and Arabidopsis thaliana. Nucleic Acids Res. 31, 5907–5916.

Iwabuchi, M., Koizuka, N., Fujimoto, H., Sakai, T., and Imamura, J. (1999) Identification and expression of the kosena radish (Raphanus sativus cv. Kosena) homologue of the ogura radish CMS-associated gene, orf138. Plant Mol. Biol. 39, 183–188.

Ogura, H. (1968) Studies on the new male sterility in Japanese radish, with special references to the utilization of this sterility towards the practical raising of hybrid seeds. Mem. Fac. Agric., Kagoshima Univ. 6, 39–78.

Park, J. Y., Lee, Y. P., Lee, J., Choi, B. S., Kim, S., and Yang, T. J. (2013) Complete mitochondrial genome sequence and identification of a candidate gene responsible for cytoplasmic male sterility in radish (Raphanus sativus L.) containing DCGMS cytoplasm. Theor. Appl. Genet. 126, 1763–1774.

Pelletier, G., Primard, C., Vedel, F., Chetrit, P., Remy, R., Rousselle, and Renard, M. (1983) Intergeneric cytoplasmic hybridization in cruciferae by protoplast fusion. Mol. Gen. Genet. 191, 244–250.

Sakai, T., and Imamura, J. (1992) Alteration of mitochondrial genomes containing atpA genes in the sexual progeny of cybrids between Raphanus sativus cma line and Brassica napus cv. Westar. Theor. Appl. Genet. 84, 923–929.

Sanchez-Puerta, M. V., Zubko, M. K., and Palmer, J. D. (2015) Homologous recombination and retention of a single form of most genes shape the highly chimeric mitochondrial genome of a hybrid plant. New Phytol. 206, 381–396.

Tanaka, Y., Tsuda, M., Yasumoto, K., Yamagishi, H., and Terachi, T. (2012) A complete mitochondrial genome sequence of Oguora-type male-sterile cytoplasm and its comparative analysis with that of normal cytoplasm in radish (Raphanus sativus L.). BMC Genomics 13, 352.

Wang, J., Jiang, J., Li, X., Li, A., Zhang, Y., Guan, R., and Wang, Y. (2012) Complete sequence of heterogenous-composition mitochondrial genome (Brassica napus) and its exogenous source. BMC Genomics 13, 675.

Yamagishi, H., and Bhat, S. R. (2014) Cytoplasmic male sterility in Brassicaceae crops. Breed. Sci. 64, 38–47.