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Note

Appearance of male sterile and black radishes in the progeny of cross between *Raphanus raphanistrum* and *Raphanus sativus*

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In addition to Ogura cytoplasmic male sterility (CMS), which is used extensively for F_1 hybrid seed production in Brassicaceae crops, two other CMS systems, NWB CMS and DCGMS, have also been identified. The causal gene for the latter two CMS systems has been identified as a novel chimeric gene, *orf463*. We previously reported that *orf463* is specific to black radish cultivars and that it is present in line 'RS-5' of *Raphanus raphanistrum*; however, the *orf463* sequence in 'RS-5' differed from that of black radish cultivars. Though, *R. raphanistrum* with an *orf463* sequence identical to that found in black radish cultivars was recently identified. We therefore sought to determine whether the *orf463* gene in line 'RS-5' induces CMS in radishes. We crossed 'RS-5' as a female parent with a cultivated radish, 'Uchiki-Gensuke', as a male parent, and examined the gross plant morphology and pollen fertility of the resulting progeny. The F_2 population contained both male sterile plants and plants with black roots. The findings showed that *R. raphanistrum* contains two types of *orf463* genes that induce CMS, and that the origin of black radishes could be attributed to *R. raphanistrum* having *orf463* gene.

Key Words: cytoplasmic male sterility, DCGMS, *orf463*, black radish, *Raphanus sativus*, *Raphanus raphanistrum*.

Introduction

Two mitochondrial genes causing cytoplasmic male sterility (CMS) have been identified in radish to date. The first gene is *orf138*, which causes Ogura CMS (Bonhomme *et al.* 1991). Ogura CMS was first discovered in Japanese radish (Ogura 1968), and the cytoplasm was introduced to *Brassica* crops. Given that the sterility associated with Ogura CMS is stable and *Brassica* crops do not have a fertility restorer gene (*Rf* gene), the Ogura CMS system was widely used in seed production for *Brassica* crops worldwide. It has been reported that *orf138* is widely distributed in Japanese wild radishes, and wild species such as *Raphanus raphanistrum* (Yamagishi and Terachi 1996, 1997).

However, most of the Korean radish cultivars possess *Rf* gene(s) for Ogura CMS. Consequently, since Ogura CMS cannot be applied to practical F_1 breeding in Korea, Korean scientists searched for other CMS systems. As a result, the NWB CMS (Nahm *et al.* 2005) and DCGMS (Lee *et al.* 2008) systems were discovered. Park *et al.* (2013) sequenced the mitochondrial genome that induced DCGMS, and identified a novel chimeric gene, *orf463*, as the causal gene; this is the second CMS gene. We also obtained the complete mitochondrial sequences of the 'Black radish' variety, and found that the genome contained *orf463* (Yamagishi *et al.* 2019). Interestingly, while *orf463* was concentrated uniquely in the black radish cultivars belonging to the 'Niger' group, one line of the wild species, *Raphanus raphanistrum*, also possessed *orf463* (Yamagishi *et al.* 2019). Recently, Wang *et al.* (2020) demonstrated that all six varieties of *Raphanus sativus* var. *niger* (Black Spanish radish) possessed *orf463*, and one line each of *R. raphanistrum* and *Raphanus maritimus* also possessed *orf463*. Furthermore, Wang *et al.* (2020) demonstrated that NWB cytoplasm has a gene that is identical to *orf463*, indicating that the same gene causes NWB CMS and DCGMS.

The cytoplasm of the four cultivars of the 'Niger' group induced male sterility in their progeny (Yamagishi *et al.* 2019). The cultivars had sequences that were identical to the *orf463* sequence determined by Park *et al.* (2013) (Yamagishi *et al.* 2019). Wang *et al.* (2020) also observed that cultivars of black Spanish radish and a line of *R. raphanistrum* have sequences that are identical *orf463* (Wang *et al.* 2020). However, the *orf463* found in *R. raphanistrum* (Yamagishi *et al.* 2019) and in

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R. maritimus (Wang *et al.* 2020) had nine nucleotide substitutions, of which seven were not synonymous.

We therefore sought to determine whether the *orf463* that we found in *R. raphanistrum* also causes male sterility. We crossed *R. raphanistrum* bearing *orf463* and the cultivar, 'Uchiki-Gensuke', that we used in our previous experiment (Yamagishi *et al.* 2019) to see if we could induce male sterility in F_2 progeny. The results showed that male sterile plants were produced in the F_2 populations. In addition, black radishes also appeared in the F_2 populations. The findings indicate that the wild species, *R. raphanistrum*, contains the genetic resources required for producing 'Niger group' cultivars and for inducing CMS due to *orf463*.

Materials and Methods

Plant materials

Two F_2 populations were produced by self-fertilizing two F_1 plants obtained from a cross between 'RS-5' (*R. raphanistrum*) and 'Uchiki-Gensuke' (hereafter, 'UC-G'). 'RS-5' was originally collected in Spain, and the seeds were stocked in the Gene Bank of Tohoku University. The seeds were kindly gifted to us by Tohoku University. Although the interspecific cross-fertility was much lower when *R. raphanistrum* was the female parent than the reciprocal cross (Yamagishi and Terachi 1997), we obtained the F_1 hybrids. Both the two F_1 plants showed normal seed fertility, producing 2.2 seeds per silique by the self-fertilization. One of the self-fertilized F_1 plants, (RS-5-5 × UC-G)-3, had red-purple root, and another one, (R-5-5 × UC-G)-4, showed green root color. All eight F_1 plants, including the two used for the self-fertilization, were male fertile, as described in our previous paper (Yamagishi *et al.* 2019). Among them, the two self-fertilized plants, (RS-5-5 × UC-G)-3 and (RS-5-5 × UC-G)-4, possessed the pollen fertility of 74.0% and 82.3%.

'UC-G' is a maintainer of Ogura CMS, and the progenies between 'UC-G' as a pollen parent and the 'Niger' group cultivars showed segregation of male sterile plants (Yamagishi *et al.* 2019). In addition, we cultivated the F_1 plants produced by a cross between 'RS-5' and 'UC-G', and the reciprocal progeny of 'UC-G' × ('RS-5' × 'UC-G'). An additional ten 'RS-5' plants were also newly cultivated and observed in this study (**Table 1**). All of the materials were cultivated in a glass house.

Observations of root surface color

The plants were cultivated in pots. Approximately two months after sowing when vegetative growth was observed, the color of the root surface was recorded in the parts above the soil (hypocotyls). Root color was classified as 'White', 'Green', 'Red-purple', or 'Black'.

Observation of pollen fertility

After each plant bloomed, we observed anther morphology and repeatedly touched the anthers with our fingers to check for pollen adhesion. As some of the progeny died before flowering, the number of plants in which pollen fertility could be observed was decreased. Plants were judged to be fertile if their anthers produced pollen, and male sterile if the pollen never adhered to the fingers. In addition, individual plants that were male sterile but which produced fertile flowers were classified as having unstable fertility. Microscopic observation of the pollen was conducted for the plants judged to be male sterile by staining with acetocarmine.

Table 1. Color of root surface in the progenies between 'RS-5' and 'UC-G'

Line or Progeny	Number of plants				
	Total	White	Green	Red-purple	Black
RS-5	10	0	7	3	0
UC-G	10	7	3	0	0
F_1 (RS-5-5 × UC-G)	6	2	1	3	0
F_2 ((RS-5-5 × UC-G)-3)	35	4	2	24	5
F_2 ((RS-5-5 × UC-G)-4)	18	5	10	0	3

DNA sequencing of orf463 in R. raphanistrum

Based on the finding that the 'RS-5' line of *R. raphanistrum* possessed *orf463* (Yamagishi *et al.* 2019), we newly cultivated the line. DNA was isolated from the leaves of each plant using a DNeasy Mini kit (Qiagen, Valencia, CA, USA) and the isolated DNA was used as a template for PCR. The PCR and the sequencing of *orf463* were performed as described in Yamagishi *et al.* (2019). We then compared the deduced protein sequences of ORF463 obtained from the sequences using the TMHMM server version 2.0 (Krogh *et al.* 2001).

Results

Root color of F_2 progeny

The root color of the wild line, 'RS-5', was green or red-purple, and the color of 'UC-G' was white or green, as shown in **Table 1** and **Fig. 1**. The F_1 plants showed variation in root color, ranging from white to red-purple (**Table 1**). In comparison, the plants of the F_2 generation varied from white to black (**Table 1**, **Fig. 1A**), and the radishes with black roots appeared in both of the F_2 populations (**Table 1**, **Fig. 1B**). The dominant root color of the F_2 populations differed depending on the F_1 plants. In one population, F_2 ((RS-5-5 × UC-G)-3), most of the plants had roots with a red-purple color, but individuals with green roots were dominant in another population, F_2 ((RS-5-5 × UC-G)-4) (**Table 1**). The major color in each of the F_2 populations corresponded to the F_1 plant from which the F_2 generation derived, respectively.

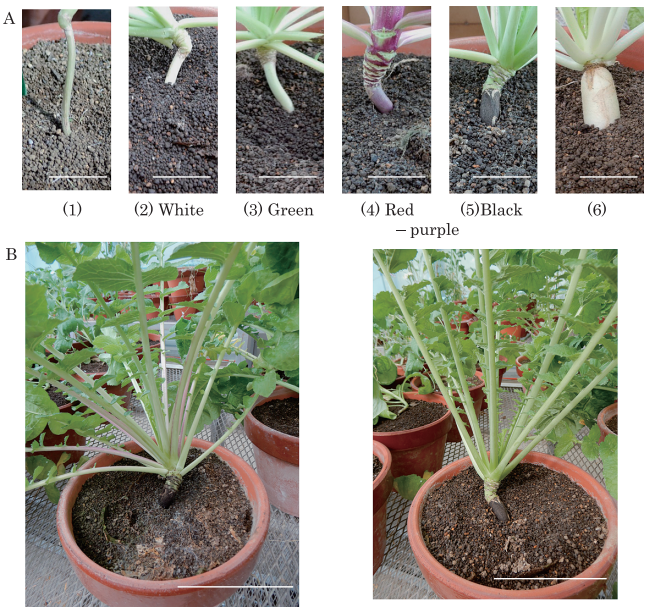

Fig. 1. Appearance of black radishes in the F_2 generation between 'RS-5' and 'UC-G'. A: Color of hypocotyl surface, (1): 'RS-5', (2) ~ (5): F_2 plants, (6): 'UC-G' (Bars indicate 3 cm). B: Black radish plants in the two F_2 populations, F_2 ((RS-5-5 × UC-G)-3) (left) and F_2 ((RS-5-5 × UC-G)-4) (right) (Bars indicate 10 cm).

Male sterility in the F_2 populations

As in our previous study (Yamagishi *et al.* 2019), all of the F_1 plants produced from a cross between 'RS-5' and 'UC-G' were male fertile (**Table 2**). The progeny between crosses of 'UC-G' as a female parent and one of the F_1 plants were fertile (**Table 2**). On the other hand, although most of the plants in the F_2 populations were fertile, male sterile plants were also observed (**Table 2**). The male sterile plants had short filaments compared to 'RS-5' and 'UC-G', and the anthers were rudimentary (**Fig. 2A**). The microscopic observations of the pollen revealed that the anthers of the sterile plants only produced deformed pollen, whereas the fertile plants produced normal pollen (**Fig. 2B**). The pollen fertility of the fertile plant shown in **Fig. 2B** was 85%, and the value was almost equal to that of 'UC-G' (88%) observed on the same day. The results showed that the fertility was restored to the level of the plants with normal cytoplasm. In addition to the fertile and sterile plants, the plants that showed unstable fertility were observed in both of the F_2 populations (**Table 2**).

Because the number of plants was not large in the two populations, the inheritance mode of pollen fertility could not be estimated. However, the results indicated that male sterility was caused by the cytoplasm of 'RS-5'. The results also demonstrated that 'RS-5' possesses *Rf* gene(s) against *orf463*. Further, the facts that the two self-fertilized F_1 plants had pollen fertility higher than 70%, and that the male sterile plants were segregated in the F_2 populations suggested that the CMS was sporophytic type.

Table 2. Male sterility in the F_2 generation between 'RS-5' and 'UC-G'

Line or Progeny	Number of plants			
	Total	Fertile	Sterile	Unstable
F_1 (RS-5-5 × UC-G)	5	5	0	0
UC-G × ((RS-5-5 × UC-G)-3)	18	18	0	0
F_2 ((RS-5-5 × UC-G)-3)	21	16	2	3
F_2 ((RS-5-5 × UC-G)-4)	14	12	1	1
RS-5	10	10	0	0
UC-G	6	6	0	0

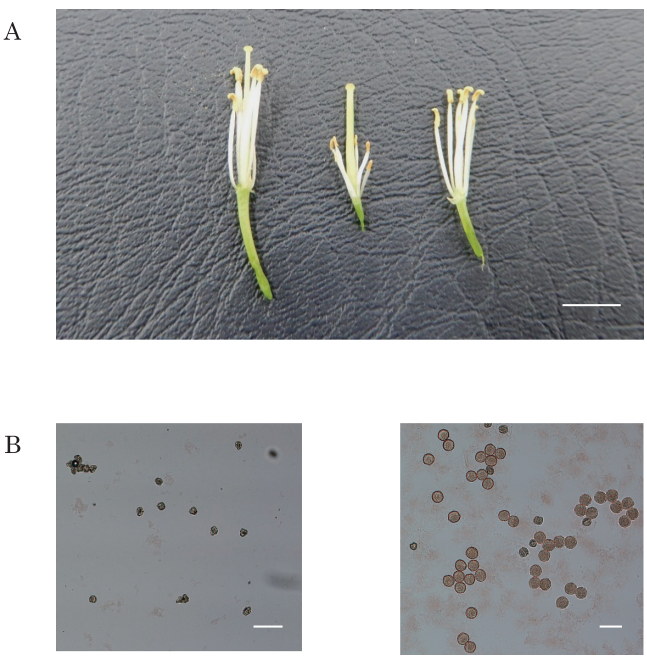

Fig. 2. Stamen and pollen of the male sterile F_2 plant between 'RS-5' and 'UC-G'. A: Stamens of 'RS-5' (left), the male sterile F_2 (center), and 'UC-G' (right) (Bar indicates 1 cm). B: Pollen grains stained with acetocarmine (left: sterile F_2 plant, right: fertile F_2 plant) (Bars indicate 100 μm).

Sequence of orf463 in 'RS-5'

The DNA sequence of *orf463* in the 10 'RS-5' plants that were newly cultivated in this experiment was identical to that observed in our previous report (Yamagishi *et al.* 2019). Compared with the *orf463* associated with DCGMS, the gene sequenced in this study contained nine nucleotide substitutions. The findings therefore indicated that all of the plants in 'RS-5' had this mitochondrial type. By the prediction of products of *orf463*, it was found that the two proteins had a similar structure and possessed 12 transmembrane domains as shown by Park *et al.* (2013) (**Fig. 3**).

Discussion

Origin of orf463 and black radish

The CMS gene, *orf463*, was first found in a collection from Uzbekistan (Lee *et al.* 2008). The observation that,

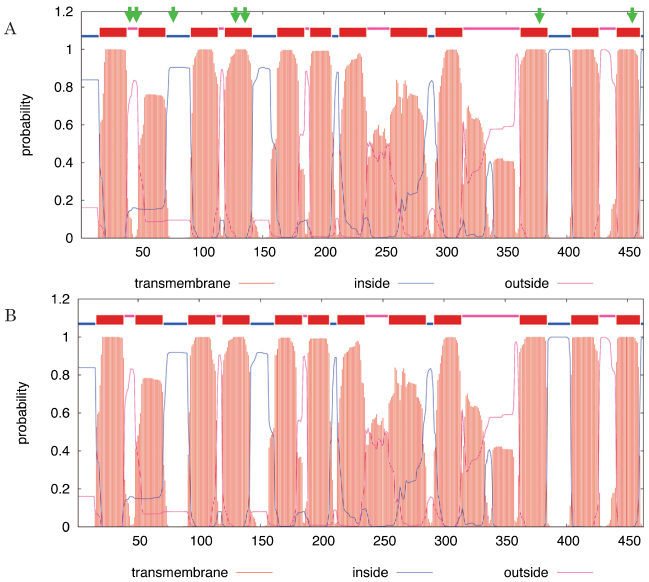

Fig. 3. Locations and probability of transmembrane domains of ORF463. A: RS-5, B: DCGMS, Arrows: Positions of amino acid substitutions.

other than male sterility, the morphology of the collection was not significantly different from that of normal radish (Lee *et al.* 2008) suggests that the accession belongs to cultivated radish. We previously found *orf463* specifically in black radishes (Yamagishi *et al.* 2019), and Wang *et al.* (2020) corroborated this finding recently. They observed that all six accessions of black Spanish radish possessed the *orf463* gene (Wang *et al.* 2020). In addition to the cultivars mentioned above, *orf463* has also been identified in *R. raphanistrum* (Wang *et al.* 2020, Yamagishi *et al.* 2019) and *R. maritimus* (Wang *et al.* 2020).

The cytoplasm of the black radishes was also shown to induce male sterility (Yamagishi *et al.* 2019). In this study, the cytoplasm of 'RS-5' was shown to cause male sterility in a similar manner to DCGMS as demonstrated by Lee *et al.* (2008). These observations indicate that the *orf463* of black radish was derived from the wild species, *R. raphanistrum*. Although the DNA sequence of 'RS-5' differs from that of black radish, Wang *et al.* (2020) found a wild radish with an *orf463* sequence that was identical to black radish.

Black roots were observed in the F_2 generation produced from a cross between 'RS-5' and 'UC-G', even though the both parents did not have black roots (**Table 1**). As reported in Nishio (2017), red and purple roots have been studied in radish cultivars; however, this is the first report to demonstrate the segregation of the black root color in progeny resulting from cross hybridization. This result also indicates the origin of black radish. Further, haplotype analysis using the chloroplast genome showed that 'RS-5' is closely related to the black radish variety (Yamagishi *et al.* 2009). Thus, based on the sharing of *orf463*, the appearance of black roots in progeny, and the similarities in the chloroplast genome, the results strongly suggest that the black radish originated from a wild species with *orf463* in their mitochondria.

Sequence variation of orf463

The DNA sequence of *orf463* in 'RS-5' was fixed to one type that had nine substitutions compared to DCGMS. Despite these differences, both types of *orf463* caused CMS. Although only two types have been found in *orf463*, nine types of *orf138* are known in the Ogura CMS gene (Yamagishi and Terachi 2001). In the case of *orf138*, the average number of DNA variations among the types was 2.28, and average number of non-synonymous nucleotide substitutions was 1.56. When we compare the size of the coding sequences of *orf138* and *orf463*, the number of nucleotide substitutions is comparable between the two CMS genes. Further, as shown in **Fig. 3**, the deduced ORF463 proteins exhibited similar characteristics despite the non-synonymous substitutions. Thus, the products of both *orf463* sequences share the same function of inducing male sterility. A more extensive search would clarify the sequence variations that exist in *orf463* in wild species, like *orf138*.

Rf genes for orf463

Lee *et al.* (2008) reported finding DCGMS, and that the inheritance pattern associated with this male sterility system varied according to parental lines. Specifically, they found that in one case a single locus was involved, and in another cross combination at least three genes were involved. Wang *et al.* (2020) also examined three crosses between a male sterile line and radish lines having *Rf* gene(s). One F_2 population exhibited the segregation of 15 fertile: 1 sterile plants, fitting an inheritance mode controlled by two loci. However, in the other two F_2 populations, no male sterile plants were observed in approximately 100 F_2 plants. In that case, at least three genes were involved. Compared to these two studies, we found the male sterile plants in the F_2 populations of smaller size, but it was difficult to estimate the inheritance mode.

In addition, we found the plants in which male fertility was unstable, the fertile or sterile phenotype differing between the flowers in an individual. This phenomenon was also observed when black radishes were used as the female parent (Yamagishi *et al.* 2019), which made it difficult to estimate the number of *Rf* genes. If the observed instability is a characteristic of the male sterility induced by *orf463*, then it would affect the practical usefulness in breeding. However, such instability was not reported in two previous articles (Lee *et al.* 2008, Wang *et al.* 2020). To better understand fertility restoration, cloning of the *Rf* genes is needed, and the DNA markers developed by Cho *et al.* (2012) would be useful for such a purpose.

Author Contribution Statement

Conceptualization, H.Y. and T.T.; Methodology, H.Y.; Investigation, H.Y., A.H. and A.F.; Writing, H.Y. and T.T.; Supervision, H.Y.

Literature Cited

Bonhomme, S., F. Budar, M. Férault and G. Pelletier (1991) A 2.5 kb *Nco*I fragment of Ogura radish mitochondrial DNA is correlated with cytoplasmic male-sterility in *Brassica* cybrids. Curr. Genet. 19: 121–127.

Cho, Y., Y.P. Lee, B.S. Park, T.H. Han and S. Kim (2012) Construction of a high-resolution linkage map of *Rfd1*, a restorer-of-fertility locus for cytoplasmic male sterility conferred by DCGMS cytoplasm in radish (*Raphanus sativus* L.) using synteny between radish and *Arabidopsis* genomes. Theor. Appl. Genet. 125: 467–477.

Krogh, A., B. Larsson, G. von Heijine and E.L. Sonnhammer (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J. Mol. Biol. 305: 567–580.

Lee, Y.P., S. Park, C. Lim, H. Kim, H. Lim, Y. Ahn, S.K. Sung, M.K. Yoon and S. Kim (2008) Discovery of a novel cytoplasmic male-sterility and its restorer lines in radish (*Raphanus sativus* L.). Theor. Appl. Genet. 117: 905–913.

Nahm, S.H., H.J. Lee, S.W. Lee, G.Y. Joo, C.H. Harn, S.G. Yang and W.B. Min (2005) Development of a molecular marker specific to a novel CMS line in radish (*Raphanus sativus* L.). Theor. Appl. Genet. 111: 1191–1200.

Nishio, T. (2017) Economic and academic importance of radish. *In*: Nishio, T. and H. Kitashiba (eds.) The Radish Genome, Springer, Switzerland, pp. 1–10.

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

Park, J.Y., Y.P. Lee, J. Lee, B.S. Choi, S. Kim and T.J. Yang (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.

Wang, Y., Q. Wang, W. Hao, J. Li, M. Qi and L. Zhang (2020) Mitochondrial genome sequencing reveals *orf463a* may induce male sterility in NWB cytoplasm of radish. Genes (Basel) 11: 74.

Yamagishi, H. and T. Terachi (1996) Molecular and biological studies on male-sterile cytoplasm in the Cruciferae. III. Distribution of Ogura-type cytoplasm among Japanese wild radishes and Asian radish cultivars. Theor. Appl. Genet. 93: 325–332.

Yamagishi, H. and T. Terachi (1997) Molecular and biological studies on male-sterile cytoplasm in the Cruciferae. IV. Ogura-type cytoplasm found in the wild radish, *Raphanus raphanistrum*. Plant Breed. 116: 323–329.

Yamagishi, H. and T. Terachi (2001) Intra- and inter-specific variations in the mitochondrial gene *orf138* of Ogura-type male-sterile cytoplasm from *Raphanus sativus* and *Raphanus raphanistrum*. Theor. Appl. Genet. 103: 725–732.

Yamagishi, H., T. Terachi, A. Ozaki and A. Ishibashi (2009) Inter- and intraspecific sequence variations of the chloroplast genome in wild and cultivated *Raphanus*. Plant Breed. 128: 172–177.

Yamagishi, H., Y. Tanaka, S. Shiiba, A. Hashimoto, A. Fukunaga and T. Terachi (2019) Mitochondrial *orf463* causing male sterility in radish is possessed by cultivars belonging to the 'Niger' group. Euphytica 215: 109.