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 Brassicaceae 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 F1 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...
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 F₂ progeny. The results showed that male sterile plants were produced in the F₂ populations. In addition, black radishes also appeared in the F₂ 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 F₂ populations were produced by self-fertilizing two F₁ 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 F₁ hybrids. Both the two F₁ plants showed normal seed fertility, producing 2.2 seeds per silique by the self-fertilization. One of the self-fertilized F₁ 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 F₁ 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 F₁ 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 morpho-

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

| Line or Progeny | Total | White | Green | Red-purple | Black |
|----------------|-------|-------|-------|------------|-------|
| RS-5           | 10    | 0     | 7     | 3          | 0     |
| UC-G           | 10    | 7     | 3     | 0          | 0     |
| F₁ (RS-5-5 × UC-G) | 6    | 2     | 1     | 3          | 0     |
| F₂ (RS-5-5 × UC-G)-3 | 35   | 4     | 2     | 24         | 5     |
| F₂ ((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 F₂ 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 F₁ plants showed variation in root color, ranging from white to red-purple (Table 1). In comparison, the plants of the F₂ generation varied from white to black (Table 1, Fig. 1A), and the radishes with black roots appeared in both of the F₂ populations (Table 1, Fig. 1B). The dominant root color of the F₂ populations differed depending on the F₁ plants. In one population, F₂ ((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, F₂ ((RS-5-5 × UC-G)-4) (Table 1). The major color in each of the F₂ populations corresponded to the F₁ plant from which the F₂ generation derived, respectively.
Male sterility in the F₂ populations

As in our previous study (Yamagishi et al. 2019), all of the F₁ 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 F₁ plants were fertile (Table 2). On the other hand, although most of the plants in the F₂ 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 F₂ 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 F₁ plants had pollen fertility higher than 70%, and that the male sterile plants were segregated in the F₂ populations suggested that the CMS was sporophytic type.

### Table 2. Male sterility in the F₂ generation between ‘RS-5’ and ‘UC-G’

| Line or Progeny                              | Total | Fertile | Sterile | Unstable |
|----------------------------------------------|-------|---------|---------|----------|
| F₁ (RS-5-5 × UC-G)                          | 5     | 5       | 0       | 0        |
| UC-G × ((RS-5-5 × UC-G)-3)                  | 18    | 18      | 0       | 0        |
| F₂ ((RS-5-5 × UC-G)-3)                      | 21    | 16      | 2       | 3        |
| F₂ ((RS-5-5 × UC-G)-4)                      | 14    | 12      | 1       | 1        |
| RS-5                                         | 10    | 10      | 0       | 0        |
| UC-G                                         | 6     | 6       | 0       | 0        |

**Fig. 1.** Appearance of black radishes in the F₂ generation between ‘RS-5’ and ‘UC-G’. A: Color of hypocotyl surface, (1): ‘RS-5’, (2) ~ (5): F₂ plants, (6): ‘UC-G’ (Bars indicate 3 cm). B: Black radish plants in the two F₂ populations, F₂ ((RS-5-5 × UC-G)-3) (left) and F₂ ((RS-5-5 × UC-G)-4) (right) (Bars indicate 10 cm).

**Fig. 2.** Stamen and pollen of the male sterile F₂ plant between ‘RS-5’ and ‘UC-G’. A: Stamens of ‘RS-5’ (left), the male sterile F₂ (center), and ‘UC-G’ (right) (Bar indicates 1 cm). B: Pollen grains stained with aceticarmine (left: sterile F₂ plant, right: fertile F₂ 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,
that all six accessions of black Spanish radish possessed the
in Nishio (2017), red and purple roots have been studied in
Although the DNA sequence of ‘RS-5’
differs from that of black radish, Wang
wild radish with an
in a similar manner to DCGMS as demonstrated by Lee
and
of black radish was derived from the wild species,
black roots in progeny, and the similarities in the chloro‐
these two studies, we found the male sterile plants in the F
2
generation produced
female parent (Yamagishi
instability is a characteristic of the male sterility induced by
orf463
in wild species, like
orf138.

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 F2 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 demon‐
strate 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 chloro-
plast 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,
ine 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 F2
population exhibited the segregation of 15 fertile: 1 sterile
plants, fitting an inheritance mode controlled by two loci.
However, in the other two F2 populations, no male sterile
plants were observed in approximately 100 F2 plants. In
that case, at least three genes were involved. Compared to
these two studies, we found the male sterile plants in the F2
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 diffi‐
cult 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.

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