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# 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

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**Abstract** The distribution of Ogura male-sterile cytoplasm among Japanese wild radish populations and Asian cultivated radishes was studied by means of polymerase chain reaction (PCR)-aided assays using mitochondrial *atp6* and *orf138* loci as molecular markers. Three separate PCR experiments were performed to amplify the target sequences in normal-type *atp6*, Ogura-type *atp6*, and Ogura-specific *orf138*, and the cytoplasm of each plant was classified as either normal or Ogura. Among 217 wild radish plants, 93 had both Ogura-type *atp6* and *orf138* (or its modified form), whereas 124 had normal-type *atp6*. Of the 93 plants with Ogura-type cytoplasm, only a single plant showed male sterility. A complete linkage between Ogura-type *atp6* and *orf138* loci was found in Japanese wild radishes, confirming our findings that Ogura-type cytoplasm is distributed widely among Japanese wild radish populations. A modified form of *orf138* (*orf138-S*) was identified in a few wild radish populations in a limited area of Japan, and the nucleotide sequence of the *orf138-S* revealed a 39-bp deletion shared in common with 'Kosena' male-sterile cytoplasm. Among the 44 Asian cultivars analyzed, 40 were determined to have normal cytoplasm since all 4 plants tested in each cultivar showed the same PCR amplification profiles as that of 'Uchiki-Gensuke', a reference cultivar with normal cytoplasm. The plants with Ogura-type cytoplasm (or its modified form) were found in 1, 1, and 2 cultivars from Tibet, Japan, and Taiwan, respectively. Except for 1 cultivar from Taiwan, those with Ogura-type cytoplasm included a few plants having male sterility. The multiple and independent introduction of Ogura-type cytoplasm from the wild radish in Asia into these cultivars is suggested.

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**Key words** *Raphanus sativus* · Wild radish · Ogura cytoplasm · Male sterility · Mitochondrial *orf138*

## Introduction

The Ogura cytoplasm, found in a Japanese radish (*Raphanus sativus* L.), is the one of the most extensively studied male-sterile cytoplasms to date. The mitochondrial genome of Ogura cytoplasm is highly rearranged relative to that of normal radish cytoplasm and contains sequences not present in the latter (Makaroff and Palmer 1988). Altered transcription patterns have been identified for at least three mitochondrial genes, *atpA*, *atp6*, and *coxI*, in the Ogura radish (Makaroff et al. 1989, 1990, 1991). Several lines of evidence have indicated that *coxI* and *atpA* are not involved in the expression of male sterility of Ogura cytoplasm (Makaroff et al. 1990, 1991). A rearrangement of *atp6*, which results in the loss of a $tRNA^{fMet}$ gene and expansion of the *atp6* open reading frame by 498 nucleotides, has been identified at the 5′ end of *atp6* in Ogura radish. However, it has been revealed that the processing of the N-terminal end of the ATP6 protein in Ogura cytoplasm yields an ATP6 core protein that is identical to that of normal radish (Krishnasamy et al. 1994). Therefore, it is unlikely that *atp6* is associated with Ogura male sterility.

On the other hand, in *Brassica* cybrids with Ogura cytoplasm, there is a close correlation between the expression of male sterility and the presence of mitochondrial 2.5-kbp *Nco*I (Bonhomme et al. 1991) and 7.0-kbp *Nru*I (Temple et al. 1992) restriction fragments. Sequencing studies on these restriction fragments revealed a new open reading frame, designated *orf138*, which is specific to Ogura cytoplasm (Bonhomme et al. 1992; Krishnasamy and Makaroff 1993). Recent studies have identified the gene product of *orf138* and support the notion that *orf138* is the actual determinant of cytoplasmic male sterility (CMS) for Ogura male sterility (Grelon et al. 1994; Krishnasamy and Makaroff 1994).

To reveal the origin of the Ogura male-sterile cytoplasm, we previously surveyed the mtDNAs of Japanese wild and cultivated radishes, both of which are *Raphanus sativus* L. A polymerase chain reaction (PCR)-aided assay using a rearrangement around an *atp6* locus was used as a molecular marker (Yamagishi and Terachi 1994a). In that study we found that wild radishes having Ogura-type *atp6* are distributed widely in Japan, whereas 15 typical Japanese cultivars possess exclusively normal-type *atp6*. We obtained the progenies of the wild radishes with Ogura-type *atp6* and the cultivars and found that male-sterile plants segregated among the progeny (Yamagishi and Terachi 1994b). We thus concluded that the original 'Ogura male sterility' (Ogura 1968) was found in a population of the wild radish or in a hybrid population between wild and cultivated radishes (Yamagishi and Terachi 1994b).

However, these findings raised another question about the origin of this cytoplasm, namely where did the Japanese wild radish possessing the Ogura cytoplasm originate? Regardless of the type of cytoplasm, the origin of Japanese wild radish itself has been somewhat controversial; it might be a form that escaped from the cultivated common radish (Makino 1909), or it might be a descendant of the wild radish that came from the Asian continent in ancient times (Aoba 1989), or both (Kumazawa 1956). Our results, however, indicated that the origin of Japanese wild radishes (at least those with Ogura-type *atp6*) could not be traced to Japanese cultivated radishes. In this context, a survey of Ogura-type cytoplasm in the Asian cultivars should provide useful information about the donor of the Ogura cytoplasm to the Japanese wild radish.

In the study described here, the validity of our earlier investigation was tested by PCR-aided assays using two mitochondrial loci, *apt6* and *orf138*, as molecular markers, because it appears that *orf138*, not *atp6*, is the acutal determinant of Ogura male sterility. To determine whether the Ogura-type cytoplasm is present in the Asian radish cultivars, we performed PCR-aided assays for *atp6* and *orf138* using various Asian radish cultivars.

The results showed a complete linkage of the Ogura-type *atp6* and *orf138* loci, confirming our findings that the Ogura-type cytoplasm is distributed widely among Japanese wild radish populations. The results also revealed a modified form of *orf138* (*orf138-S*) in a few wild radish populations in a limited area of Japan and that there is Ogura-type cytoplasm in a few Asian cultivars.

## Materials and methods

### Plant materials

Table 1 lists the 217 wild radish plants studied. These were collected from 26 populations located at various sites in Japan (Fig. 1). Of these, 21 had been used in our previous study (Yamagishi and Terachi 1994a), whereas 5 populations, Iriomote, Zanpazaki, Tanesashi, Erimomisaki, and Soekawa, were new. The plants from these 5 populations were provided by H. Yamaguchi, the Faculty of Agriculture, Osaka Prefectural University, Japan. 'Soekawa' is an inland area of Yamagata Prefecture, and the plants growing in this area are called Nora-daikon in Japanese; the other 4 new populations are located near the coast (hence, these plants are called Hama-daikon in Japanese). Two to 12 plants per population were analyzed.

Table 2 shows the 44 Asian cultivars used in the study. They were provided by the National Research Institute for Vegetables, Ornamental Crops and Tea (NIVOT), Mie, Japan, the National Institute of Agrobiological Resources (NIAR), Ibaragi, Japan, and S. Yazawa, the Faculty of Agriculture, Kyoto University, Japan. Thirty of the cultivars were introduced from various regions of China, while 1, 2, 3, and 5 cultivars were of Indian, Thai, Russian, and Korean origin, respectively (Fig. 2). The remaining 3 cultivars are Japanese in origin, of which 'Kosena' has male-sterile cytoplasm (Ikegaya 1986b). Four plants per cultivar were analyzed. Single 'Uchiki-Gensuke' (normal cytoplasm) and 'MS-Gensuke' (Ogura cytoplasm) plants were used as references.

The seeds of these plants were sown in pots, and the plants were grown in a greenhouse. After harvesting one leaf for DNA isolation, each plant was allowed to grow until flowering, and fertility was evaluated by visual examination of anther development.

### Total DNA isolation and the PCR-aided assays of mtDNAs

Total DNA was isolated from a young leaf of individual plants as described (Yamagishi and Terachi 1994a). The *atp6* locus was examined by PCR using primers A, B, and C (Fig. 3a) (Yamagishi and Terachi 1994a). Briefly, primer A is located in *trnfM* of normal-type mtDNA, while primer B is a 20 mer present in duplicate at 63–82 bp and 135–154 bp of *atp6* in the Ogura-type mtDNA. Primer C is located commonly in both types of *atp6*. PCR with primers A and C should amplify a 331-bp DNA fragment in normal mtDNA, whereas 543-bp and 471-bp fragments should be generated in Ogura-type mtDNA after PCR using primers B and C.

To amplify Ogura-specific *orf138*, we used the following oligonucleotides as primers: primer D, 5'GACATCTAGAAAGTTAAAAAT3'; primer E, 5'AGCAATTGGGTTCACAAAGCAT3'. The former is a sense 22 mer corresponding to position 161–182 in the coding region of *orf138*, whereas the latter is an antisense 22 mer located immediately 3' to the gene (Fig. 3b). Thirty PCR cycles were carried out in a PC-700 programmable incubator. Each cycle consisted of denaturation for 1 min at 94 °C, annealing for 1 min at 52 °C, and extension for 2 min at 72 °C. The other conditions were the same as those described by Yamagishi and Terachi (1994a). PCR using primers D and E amplifies a 278-bp DNA fragment in plants with Ogura-type mtDNA, whereas no PCR product appears in plants with normal-type mtDNA. Amplified DNA fragments were separated by electrophoresis on a 3% Nusieve (FMC BioProducts, USA) agarose gel.

### Nucleotide sequencing of the *orf138-S* fragment

To elucidate the relationship between *orf138-S* and *orf138*, we determined the nucleotide sequence of the amplified DNA fragment by nonradioactive sequencing. For this purpose, *Taq* DNA polymerase (Promega, USA) was used in the PCR with primers D and E. The other PCR conditions were the same as those already described. The amplified DNA fragment was cloned into an *Eco*RV site of pBluescriptII SK$^+$ (Stratagene, USA), which was modified to a 'T-vector' according to the method of Marchuk et al. (1990). Double-stranded plasmid DNAs were isolated by a standard alkaline lysis procedure (Maniatis et al. 1982). Cycle sequencing was performed using a ΔTth DNA polymerase sequencing PRO kit (Toyobo, Japan) in combination with Digoxigenin-labeled M13/pUC sequencing and reverse sequencing primers (Boehringer, Germany). The DNA fragments were separated by 6% polyacrylamide gel electrophoresis, then transferred to a Hybond-N membrane (Amersham, UK) by contact blotting. Sequencing ladders were detected by an anti-Digoxigenin antibody conjugated to alkaline phosphatase using the chemiluminescent substrate Lumigen PPD (Wako, Japan) following the protocols

**Table 1** Mitochondrial DNA types of wild radish populations detected by PCR using normal and Ogura-specific *atp6* primers and Ogura-specific *orf138* primers

| Number | Abbreviation | Population | Number of plants | *atp6* | | *orf138* |
|---|---|---|---|---|---|---|
| | | | | Normal | Ogura | |
| 1 | Irm | Iriomote | 12 | 10 | 2 | 2 |
| 2 | Znp | Zanpazaki | 10 | 4 | 6 | 6 |
| 3 | Okn | Okinoerabu | 3 | 3 | 0 | 0 |
| 4 | Tng | Tanegashima | 4 | 0 | 4 | 4 |
| 5 | Ksk | Kushikino | 2 | 0 | 2 | 2 |
| 6 | Tmk | Tomioka | 12 | 1 | 11 | 11 |
| 7 | Fke | Fukue | 4 | 2 | 2 | 2 |
| 8 | Imj | Imajuku | 12 | 5 | 7 | 7 |
| 9 | Ngt | Nagato | 4 | 4 | 0 | 0 |
| 10 | Nim | Nima | 4 | 4 | 0 | 0 |
| 11 | Dai | Daiei | 12 | 6 | 6 | 6 |
| 12 | Hms | Hamasaka | 3 | 1 | 2 | 2 |
| 13 | Hkt | Hikatae | 12 | 11 | 1 | 1 |
| 14 | Kkz | Kakizaki | 12 | 5 | 7 | 7 |
| 15 | Mjm | Majima | 4 | 4 | 0 | 0 |
| 16 | Atm | Atsumionsen | 12 | 4 | 8 | 8 (7)[a] |
| 17 | Nkh | Nikaho | 12 | 12 | 0 | 0 |
| 18 | Iws | Iwasaki | 12 | 5 | 7 | 7 (7)[a,b] |
| 19 | Kss | Kasose | 4 | 4 | 0 | 0 |
| 20 | Srh | Shirahama | 12 | 2 | 10 | 10 |
| 21 | Nth | Natahama | 4 | 1 | 3 | 3 |
| 22 | Mhm | Mihama | 12 | 10 | 2 | 2 |
| 23 | Cts | Chitose | 12 | 2 | 10 | 10 |
| 24 | Tns | Tanesashi | 4 | 1 | 3 | 3 |
| 25 | Erm | Erimomisaki | 9 | 6 | 3 | 3 |
| 26 | Skw | Soekawa (Nora-daikon) | 14 | 14 | 0 | 0 |
| | | Total | 217 | 124 | 93 | 93 |

[a] Numbers in parentheses indicate plants with a DNA band of smaller molecular weight than that predicted from the design of the primers
[b] One of the 7 plants showed male sterility, whereas the other 216 plants were male-fertile

provided in the DIG *Taq* DNA sequencing kit (Boehringer, Germany).

## Results

### The distribution of Ogura-type cytoplasm among Japanese wild radishes

Figure 4 shows the amplified DNA fragments of the two reference cultivars 'Uchiki-Gensuke' and 'MS-Gensuke' after three separate PCR amplifications. 'Uchiki-Gensuke', which is a maintainer of Ogura male sterility, yielded a normal *atp6*-specific 331-bp fragment upon PCR analysis using primers A and C, whereas no amplified DNA fragment appeared upon PCR analysis using the B and C primer pairs. On the other hand, 'MS-Gensuke', a male-sterile cultivar carrying Ogura cytoplasm, generated 543-bp and 471-bp DNA fragments of Ogura *atp6* after PCR using primers B and C, and a 278-bp DNA fragment of *orf138* by PCR primers D and E. In this cultivar, no amplified DNA fragment appeared upon PCR with primers A and C.

Table 1 summarizes the results of three PCR amplifications performed on 217 individual wild radish plants. Ninety-three plants yielded both the 543-bp and 471-bp DNA fragments from the *atp6* locus, which was the result expected in Ogura radish using primers B and C. In contrast, 124 plants yielded a 331-bp fragment upon PCR using primers A and C, thereby reflecting normal *atp6*. All the plants yielded the predicted amplification products in one of the two PCR experiments. The amplification profile of the *orf138* locus in each plant was strictly correlated with that of the *atp6* locus; all 93 plants with Ogura-type *atp6* generated an amplification product of *orf138* after PCR using primers D and E, whereas there was no amplification product in the 124 plants having normal-type *atp6*.

In our previous survey of 4 plants per population, plants with Ogura-type *atp6* were found in 14 of 24 populations. In addition, 9 of these 14 populations contained both plants of normal- and Ogura-type *atp6* (Yamagishi and Terachi 1994a). In this study we analyzed 26 populations using two mitcohondrial markers, *atp6* and *orf138*, and far more plants were assayed (Table 1). Consequently, in 17 of the 26 populations, individual plants were identified with either normal- or Ogura-type mtDNA. Two populations (Tanegashima and Kushikino) consisted only of plants with Ogura-type mtDNA, whereas the remaining 7 populations (including Soekawa) contained only those with normal-type mtDNA. These results indicate that Japanese wild

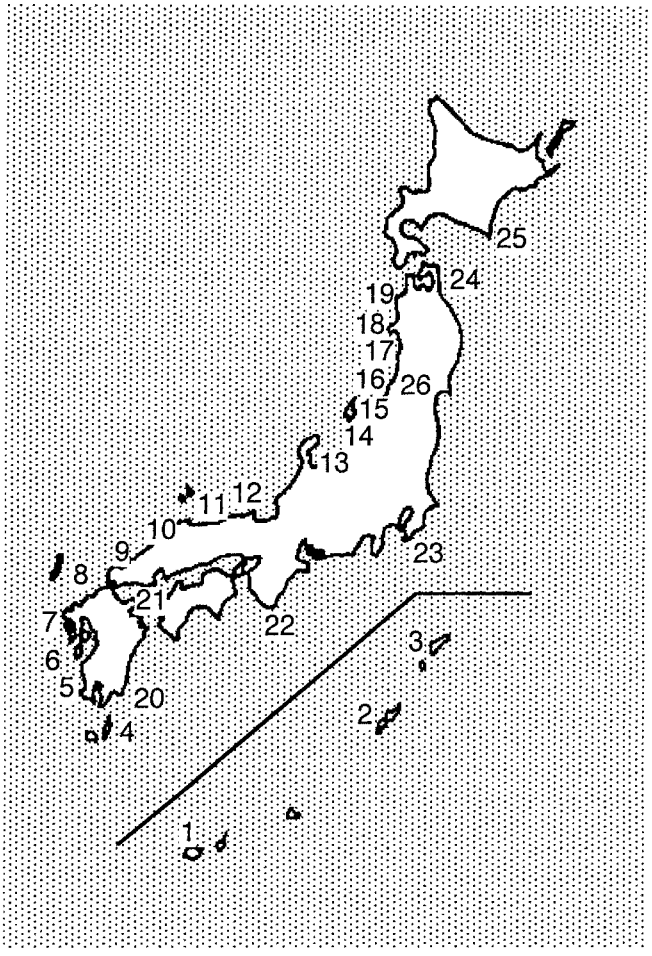



**Fig. 1** Map of Japan with the collection sites for wild radish (*Raphanus sativus*) numbered as given in Table 1

radish populations, especially those located near the coast, generally contain plants with Ogura-type cytoplasm and that plants with Ogura- and normal-type cytoplasm grow sympatrically in most of these populations.

## A modified form of *orf138* found in Japanese wild radishes and the cultivar 'Kosena'

Among the 93 plants showing the *orf138* PCR product, 79 yielded a 278-bp DNA fragment that reflects Ogura mtDNA; the remaining 14 plants from 2 populations of the Tohoku district (Atsumionsen and Iwasaki) yielded a DNA fragment smaller than that predicted (designated *orf138-S*, Fig. 5). In the Iwasaki population, all 7 plants with Ogura-type *atp6* yielded *orf138-S*, whereas in the Atsumionsen population 7 of 8 plants with Ogura-type *atp6* had it (Table 1). This smaller fragment was also observed in the Japanese cultivar 'Kosena', which is described below. One plant with *orf138-S* in the Iwasaki population exhibited male sterility under our growth conditions; it was the only plant demonstrating male sterility among the 217 wild radish plants analyzed.

Figure 6 shows the nucleotide sequence of an *orf138-S* fragment from wild radish obtained by PCR using primers D and E. The sequences were determined for 1 and 5 plants of the Atsumionsen and Iwasaki populations, respectively, and they were identical. Sequencing analyses of *orf138-S* revealed that this fragment exhibited a 39 nucleotide deletion in the coding region of *orf138*. The deleted sequence was either the middle or the last repeat of the three tandemly repeated sequences coding a lysine and glutamic acid-rich 13 amino acid Motif (Krishnasamy and Makaroff 1994). Iwabuchi et al. (1993) determined the sequence of *orf138* in 'Kosena' and found the deletion of 39 nucleotides, designating the open reading frame of 'Kosena' as *orf125* instead of *orf138*. We also analyzed the sequence of a single plant of 'Kosena' and observed that it had the identical deletion with *orf138-S* of the wild radishes mentioned above. From these results, although we did not sequence the entire *orf138-S*, it is suggested that the *orf138-S* of the wild radishes was created by a deletion of 39 nucleotides near the 3′ terminus of the gene.

## Ogura-type cytoplasm in Asian cultivars

The mtDNAs of 176 individual plants derived from 44 Asian cultivars were assayed. Among those, 40 were judged to have a normal cytoplasm since all of the plants yielded a 331-bp DNA fragment of normal *atp6* upon PCR using primers A and C. The fact that no amplification product was obtained from these 40 cultivars by PCR with primers B and C (for Ogura-type *atp6*) or D and E (for *orf138*) supported this conclusion (Table 2). The results, together with our previous observation of 15 Japanese cultivars (Yamagishi and Terachi 1994a), indicated that most Asian radish cultivars have normal-type cytoplasm.

The four remaining cultivars, however, consisted of plants with Ogura-type cytoplasm (Table 3). All 4 plants of each of cvs 'Daibaika' and 'Soubaika Kansaitou' and the 2 plants of cv 'Chibettokei Daikon' yielded the same amplification products of *atp6* and *orf138* as those with Ogura cytoplasm. The remaining cultivar, 'Kosena', which is native to the Tohoku district, also consisted of 2 plants possessing an Otura-type *atp6* locus. However, in 'Kosena' with Ogura-type *atp6*, the product of *orf138* was of the *orf138-S* type. This fragment was the same as that found in the wild radishes.

Anther development showed that both plants in 'Kosena' simultaneously carrying Ogura-type *atp6* and *orf138-S* exhibited male sterility. Similarly, 1 and 2 male-sterile plants were identified in each of 'Chibettokei Daikon' and 'Daibaika' among 2 and 4 plants judged to have an Ogura-type cytoplasm in the former and latter cultivars, respectively. The remaining 2 and 3 plants in 'Daibaika' and 'Chibettokei Daikon' were fertile irrespective of the cytoplasm. Further, there were no male-sterile plants in 'Soubaika Kansaitou', although all 4 plants had both the Ogura-type *atp6* and

**Table 2** Mitochondrial DNA types of Asian radish cultivars

| Code | Variety name | Origin[a] | Source[b] | mtDNA type[c] | Code | Variety name | Origin[a] | Source[b] | mtDNA type[c] |
|---|---|---|---|---|---|---|---|---|---|
| 88-1 | Genpaku Roobo | C | A | N | 93-61 | Daikouhou | C | B | N |
| 88-20 | Dooshi Roobo | C | A | N | 93-62 | Jyoutou Sei | C | B | N |
| 89-222 | Roroubo 2 | C | A | N | 93-63 | Ibou Ao | C | B | N |
| 89-352 | Saksa | K | A | N | 93-64 | Keijou Arutari | K | B | N |
| 89-353 | Teplichniy Gribovskiy | R | A | N | 93-65 | Kinmon Akamaru | K | B | N |
| 89-354 | Mokhovskiy | R | A | N | 93-68 | Nanyou Natsumaki Daikon 30 | C | B | N |
| 91-118 | Red with White Tip | C | A | N | 93-69 | Okinawa Zairai | J | B | N |
| 91-354 | Yuan Bai Luobo | C | A | N | 93-70 | Pusa Desi | I | B | N |
| 92-45 | 40 Ten Tian Zao Loubo | C | A | N | 91-71 | Ro Hachibu | C | B | N |
| 92-487 | Xiao Shui Luobo | C | A | N | 93-72 | Ryuu Ken | K | B | N |
| 92-496 | Da Hong Pao | C | A | N | 93-73 | Shinshuu Ootsubo | K | B | N |
| 93-214 | Shiki Roobo (Kon) | C | A | N | 93-74 | Shoukeisui Ryuusui Daikon | C | B | N |
| 93-216 | Chibetto Kei Daikon | C | A | N + O | 93-75 | Soubaika Kansaitou | C | B | O |
| 93-217 | Adana Kei Daikon | C | A | N | 93-76 | Waincha | J | B | N |
| 93-218 | Akamaru Daikon | C | A | N | 93-77 | Zolotisto Zheltaja | R | B | N |
| 93-219 | Shiromaru Daikon | C | A | N | 93-66 | Kosena | J | B | N + S |
| 93-220 | Eruzurumu Kei Daikon | C | A | N | 93-67 | Koshuuban | C | B | N |
| 93-221 | Seizou Kougen Daikon | C | A | N | 94-10 | Rats Tail-1 | T | C | N |
| 93-222 | Seizou Dai Daikon | C | A | N | 94-11 | Rats Tail-2 | T | C | N |
| 93-223 | Seizou Daikon (Sei) | C | A | N | 94-12 | Shiki Daikon | C | C | N |
| 93-58 | Baika Shiro | C | B | N | 94-13 | Abura Daikon | C | C | N |
| 93-59 | Bein Roubu | C | B | N | C-16 | Uchiki-Gensuke | | D | N |
| 93-60 | Daibaika | C | B | O | C-17 | MS-Gensuke | | E | O |

[a] C, China; I, India; J, Japan; K, Korea; R, Russia, T, Thailand
[b] A, NIVOT; B, NIAR; C, Dr. S. Yazawa; D, Takii Seed Co. Ltd.; E, Dr. M. Yamabe
[c] N, All the plants yielded the PCR product of normal-type *atp6*, but not Ogura-type *atp6* nor *orf138*; O, All the plants yielded the PCR products of Ogura-type *atp6* and *orf138*; N + O, the plants of the preceding two types were present in one cultivar; N + S, the plants yielding normal *atp6* and those yielding Ogura-type *atp6* and *orf138-S* were present in a cultivar

*orf138*. The total number of plants with Ogura-type *atp6* and *orf138* (including *orf138-S*) in the cultivars was 12, and 5 of these exhibited male sterility. This high frequency of male-sterile plants is in marked contrast with that found in Japanese wild radishes (1 of the 93 Ogura-type plants).

## Discussion

### The linkage between Ogura-type *atp6* and *orf138* loci in Japanese wild radishes

Using primers A and C, or B and C, we were able to classify the mitochondrial *atp6* locus in radish as either normal or Ogura-type. Similarly, PCR using primers D and E definitely identifies the Ogura-specific *orf138* locus (Fig. 4). We found a complete linkage between Ogura-type *atp6* and *orf138* loci in Japanese wild radishes. All of the plants with Ogura-type *atp6* possess *orf138* (or its modified form), whereas all those with normal *atp6* lack *orf138*. Therefore, our finding that wild radishes with Ogura-type cytoplasm are present in various populations in Japan is valid, though we used *atp6* as a molecular marker of mtDNA (Yamagishi and Terachi 1994a).

The present results also indicate that the mtDNA configuration originally demonstrated in Ogura male-sterile radish (Makaroff and Palmer 1988) is present in some wild radishes. According to comparative restriction mapping of mtDNAs (Makaroff and Palmer 1988), the organization of mitochondrial genome is highly divergent between normal and Ogura radish. Two mtDNAs differed in arrangement by at least ten inversions, suggesting the earlier divergence of the two cytoplasms. It should be noted, however, that we observed the sympatric growing of wild radishes with Ogura-type and normal-type cytoplasm. Despite the fact that a small sample was studied (2–12 plants per population), two-thirds (17/26) of the wild radish populations consisted of plants with Ogura- and normal-type cytoplasm. This suggests a diphyletic origin of the Japanese wild radish.

### *Orf138-S* in Japanese wild radishes and origin of 'Kosena' male-sterile cytoplasm

PCR using primers D and E generated two *orf138* products, namely the 278-bp fragment in the Ogura male-sterile cytoplasm and a smaller DNA fragment, designated *orf138-S*. The latter fragment was found in wild radishes from 2 populations in the Tohoku district, Iwasaki and Atsumionsen, and in 1 Japanese cultivar, 'Kosena', which is native to Tohoku. Some of the radish plants among the Iwasaki population and Kosena with

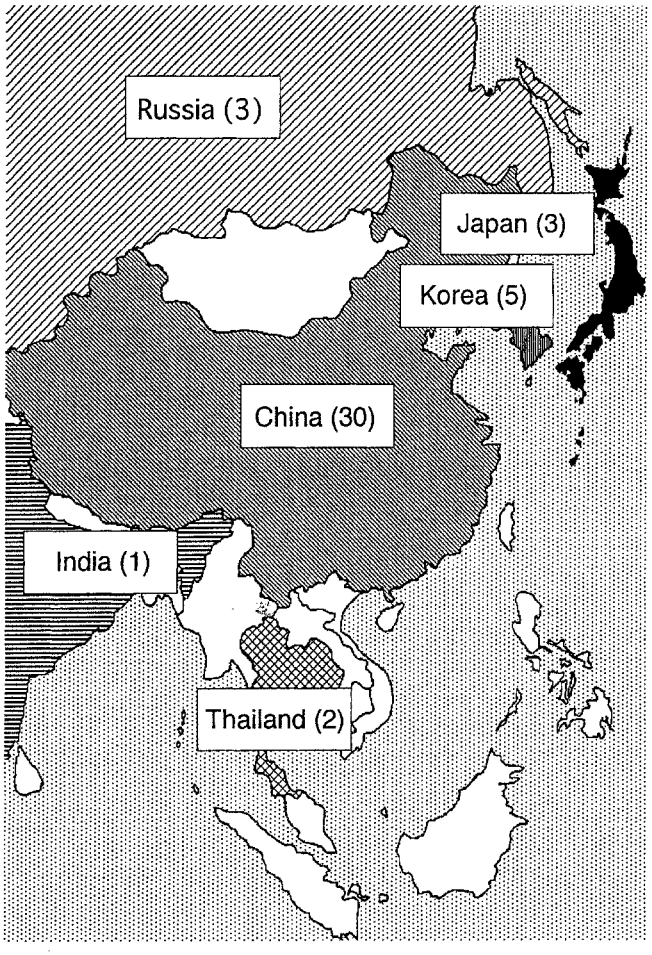



**Fig. 2** Distribution of Asian cultivars of the radish (*Raphanus sativus*) used in the study. *Numbers* in *parentheses* show the number of cultivars studied

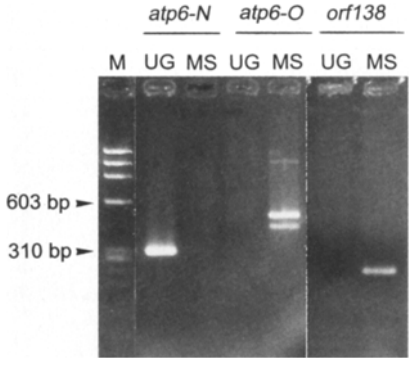



**Fig. 4** Amplification of DNA fragments by PCRs using primers A and C (*atp6-N*), B and C (*atp6-O*), and D and E (*orf138*) in radish cultivars used as a reference with normal ('Uchiki-Gensuke', *UG*) and Ogura ('MS-Gensuke', *MS*) cytoplasm. *M* indicates the $\phi$X174/*Hae*III digest used as a molecular size marker

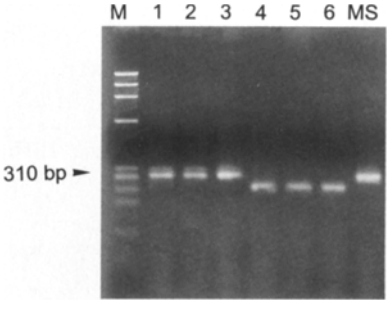
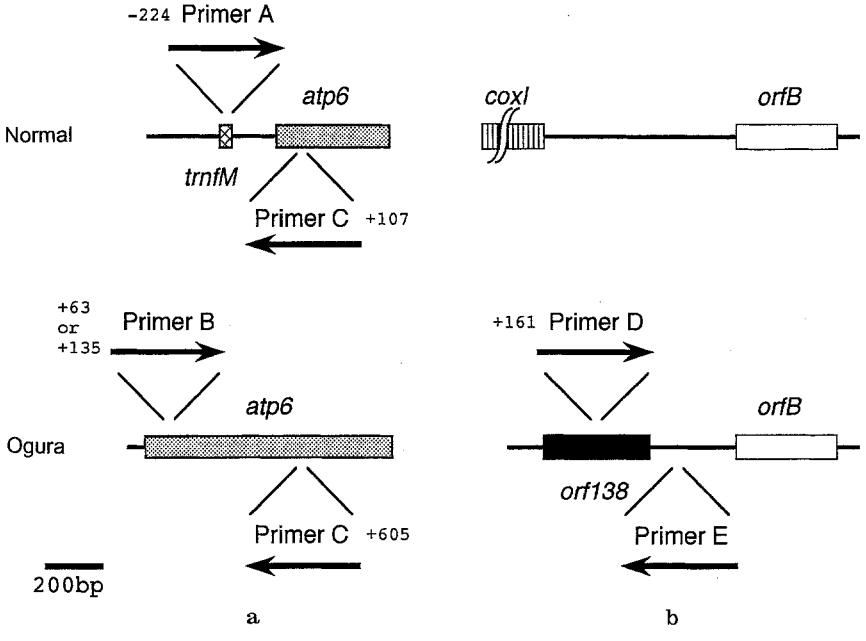



**Fig. 5** An *orf138-S* DNA fragment (*lanes 4,5* and *6*) found in 'Kosena' and some wild radish plants. An *orf138* DNA fragment of 278 bp (*lanes 1,2,3* and *MS*) is shown for comparison. The following DNA templates were used: *1* 'Chibetto Kei Daikon', *2* 'Daibaika', *3* 'Soubaika Kansaitou', *4* 'Kosena', *5* Iwasaki, *6* Atsumionsen, *MS* 'MS-Gensuke' *M* indicates the $\phi$X174/*Hae*III digest used as a molecular size marker

**Fig. 3a,b** Mitochondrial loci studied by PCR-aided assays to classify radish cytoplasm. The relative positions of the primers are indicated on the *atp6* (**a**) and *orf138*/*orfB* (**b**) loci of mtDNAs from normal and Ogura radishes (after Krishnasamy and Makaroff 1993 and Krishnasamy et al. 1994)



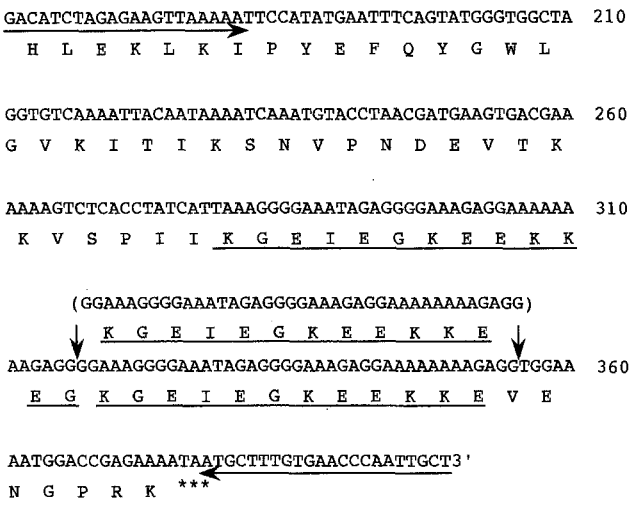



**Fig. 6** Nucleotide sequence of an *orf138-S* DNA fragment of wild radish. *Horizontal arrows* indicate the sequences corresponding to the PCR primers (D and E). The 39 nucleotides shown between *parentheses* are deleted at one of the two points indicated by the *vertical arrows*. Amino acids tandemly repeated three times in *orf138* are *underlined* (after Krishnasamy and Makaroff 1994). The *numbers* refer to the location relative to the start codon of *orf138*

the *orf138-S* exhibited male sterility under our growth conditions. This finding agrees with the report of Ikegaya (1986b) in which 'Kosena' male-sterile cytoplasm was first documented. From the similarity in the morphology and geographical distributions and the identity of *orf138-S* sequences, we propose that the 'Kosena' male-sterile cytoplasm (Ikegaya 1986b) is derived from Japanese wild radishes with *orf138-S*.

Iwabuchi et al. (1993) have identified a 39-bp deletion in a putative coding region of *orf138* in a 'Kosena' mtDNA. Our sequencing data revealed that *orf138-S* in the wild radish plants possess the same deletion as that found in 'Kosena'. The location of the deletion corresponds to the hydrophilic region tandemly repeated three times in ORF138 protein (Krishnathamy and Makaroff 1994). Since some radish plants with *orf138-S* show male sterility, how the deletion influences the function of *orf138* warrants further investigation.

## Ogura-type mtDNA found in Asian cultivars

PCR assays indicated that most of the Asian cultivars analyzed here had normal mtDNA. In terms of the *atp6* locus, all 15 Japanese radish cultivars analyzed by Yamagishi and Terachi (1994a) also have normal mtDNA. However, we found plants with Ogura-type mtDNA in the 3 Asian radish cultivars, 'Daibaika', 'Soubaika Kansaitou', and 'Chibetto Kei Daikon', as well as in 1 Japanese cultivar, 'Kosena'.

The first two Asian cultivars belong to the group Baika and have a similar root shape. This cultivar group reportedly originated in South China and is now distributed across Indo-China, Taiwan and the southern islands of Japan (M. Ashizawa, personal communication). In Taiwan, a male-sterile plant has been found in a radish cultivar whose female parent was 'Daibaika' (Ikegaya 1986a). This and our results indicate that the cultivar group Baika includes cytoplasm that can induce male sterility in radish just like the Ogura cytoplasm under certain nuclear backgrounds. The relationship between the plants in the Baika group and Japanese wild radish with Ogura-type cytoplasm is not clear. Further characterization of the plants in the Baika group is required.

'Chibettokei Daikon' was introduced from China to NIVOT as a variety grown in Tibet. This variety had relatively small roots and leaves, and the root is hard and not as juicy as the Japanese varieties. These characteristics are the same as those of the radish cultivars native to the Tibetan region, which the first author observed in a mountainous area of Nepal.

It is notable that cultivars with Ogura-type mtDNA (or its modified form) have been found in diverse geographical regions in Asia (Tibet, South China and Japan) and that they have a very different morphology. It is difficult to assume that these cultivars with Ogura-type mtDNA had an immediate common ancestor. Rather, the results indicate a broader distribution of the wild radish with Ogura-type cytoplasm than only Japan and the multiple and independent introduction of the Ogura-type cytoplasm from a wild radish to cultivars in diverse areas of Asia. Further studies including the Asian wild radish and wild relatives of radish should clarify this point.

**Table 3** Frequencies of plants having the Ogura-type mtDNA and showing the phenotype of male sterility in the varieties including the Ogura-type

| Variety | Origin | Number of plants analysed | Number of plants with Ogura-type mtDNA | Male-sterile plants |
|---|---|---|---|---|
| Chibetto Kei Daikon | Tibet | 4 | 2 | 1 |
| Daibaika | Taiwan | 4 | 4 | 2 |
| Soubaika KIansaitou | Taiwan | 4 | 4 | 0 |
| Kosena | Japan | 4 | (2)[a] | 2 |
| | Total | 16 | 12(2)[a] | 5 |

[a] Two Kosena plants showed a DNA band of smaller molecular weight (*orf138-S*) than that predicted from the design of the primers in *orf138*

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