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# Molecular and biological studies on male-sterile cytoplasm in the Cruciferae. I. The origin and distribution of Ogura male-sterile cytoplasm in Japanese wild radishes (*Raphanus sativus* L.) revealed by PCR-aided assay of their mitochondrial DNAs

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**Abstract.** Ogura male-sterile cytoplasm was surveyed in common Japanese radish cultivars and in wild radishes growing in various localities in Japan. Mitochondrial (mt) DNA rearrangement involving the *atp6* gene was used as a molecular marker. To detect the mtDNA rearrangement, polymerase chain reactions (PCR) were designed to amplify the upstream region of the *atp6* gene. The oligonucleotides homologous to the following three regions were synthesized: (1) *trnfM*, (2) *ORF105* and (3) *atp6*. PCRs were conducted with a pair of the first and the third primers to detect normal mtDNA, and with the second and the third primers for Ogura-type mtDNA. All 15 Japanese cultivars yielded an amplification product which was the same as that of normal mtDNA, whereas some wild radishes gave the product specific to Ogura mtDNA. Twenty-four populations of wild radish were classified into three groups according to the frequency of Ogura-type mtDNA: (1) in ten populations, all four plants analyzed per population had normal type mtDNA, (2) in five populations, only plants with Ogura-type mtDNA were found, and (3) nine populations included both normal and Ogura-type mtDNAs. There were no geographical restrictions and no cline in the distribution of the plants with Ogura-type mtDNA. These results suggested that the Ogura-type male-sterile cytoplasm originated in wild radishes.

**Key words:** *Raphanus sativus* – wild radish – Mitochondrial DNA – Polymerase chain reaction – Ogura male sterility

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## Introduction

Although self-incompatibility is mainly employed in Cruciferous crops for $F_1$ hybrid production, several male-sterility systems have been explored for practical use (Shiga 1980). Among them, the cytoplasmic male sterility based on Ogura cytoplasm (Ogura 1968) has been used in the radish (Bonnet 1977), as well as in *Brassica* crops to which Ogura cytoplasm was introduced by intergeneric hybridization or cell fusion (Bannerot et al. 1977; Pelletier et al. 1983; Kao et al. 1991). In his original publication Ogura (1968) described the origin of the cytoplasm as follows: 'a male-sterile radish strain obtained from an unidentified variety, at Onejime, Kagoshima prefecture'. Thus in a strict sense, the origin and distribution of this cytoplasm remain poorly understood, despite its world-wide use.

Recently, Makaroff and Palmer (1988) compared the organization of mitochondrial (mt) DNA from Ogura cytoplasm with that from normal cytoplasm, and found that rearrangements, including each of the *atpA*, *atp6* and *coxI* genes, occurred between Ogura and normal mtDNAs (Makaroff et al. 1989, 1990, 1991). Their findings of mtDNA rearrangements between Ogura and normal cytoplasm led us to investigate the origin and distribution of Ogura cytoplasm in Japan, since the two types of cytoplasm are distinguishable using the rearrangements as molecular markers. The polymerase chain reaction (PCR) (Saiki et al. 1985) is the method of choice for the purpose; using a pair of primers specific to Ogura and normal mtDNA, a large number of plant samples can be analyzed individually without either labor-intensive test crosses or phenotypic observations of fertility.

Wild radishes grow spontaneously in various areas of Japan, especially along the coast. If an Ogura-speci-

**Table 1.** Radish cultivars used in the present analysis

| Code | Cultivar name | Source[a] | Code | Cultivar name | Source[a] |
|---|---|---|---|---|---|
| C-01 | Awa-shin-bansei | A | C-10 | Akita | B |
| C-02 | Momoyama | B | C-11 | Natumino | B |
| C-03 | Shinsyuu-Jidaikon | B | C-12 | Houryou | A |
| C-04 | Minowase | C | C-13 | Aonaga | A |
| C-05 | Oosaka-Shijuunichi | B | C-14 | Shunjuu-Arutari | B |
| C-06 | Ookura | A | C-15 | Icicle | A |
| C-07 | Shiroguki-Kameido | B | C-16 | Uchiki-Gensuke | A |
| C-08 | Kibakei-Riso | A | C-17 | MS-Gensuke | D |
| C-09 | Ninengo | B | | | |

[a] A, Takii Seed Co. Ltd.; B, NIVOT; C, Oohara Seed Co. Ltd.; D, Dr. M. Yamabe

fic mtDNA could be found among these Japanese radishes by PCR, the distribution of that cytoplasm would be clarified. In this report, as a first step toward a full understanding of the origin and distribution of Ogura cytoplasm, we surveyed Ogura-type mtDNA both in cultivars and in Japanese wild radishes using PCR-aided assays designed to amplify a rearranged region including the *atp6* gene as a target. The results demonstrated that Ogura-type mtDNA is present in wild radish populations of various areas of Japan, whereas no cultivars studied here had Ogura-type mtDNA.

## Materials and methods

*Plant materials*

Table 1 shows the 17 cultivars of *Raphanus sativus* L. analyzed in this study. Most of them belong to main Japanese radish cultivar groups. The cultivars were provided by NIVOT (National Research Institute for Vegetables, Ornamental Crops and Tea, Japan) or else were purchased from private seed companies. As a control for Ogura mtDNA, 'MS-Gensuke' was used; this was bred by M. Yamabe, of Ishikawa Agricultural Research Institute, by introducing Ogura male-sterile cytoplasm into 'Uchiki-Gensuke'.

Table 2 lists the 24 wild radish populations studied. Their collection was made by O. Ohnishi, the Faculty of Agriculture, Kyoto University, Japan.

Four individual plants per cultivar or per population were analyzed. These plants were sown in pots, grown in a greenhouse, and the total DNA was then isolated.

**Table 2.** The wild radishes used in the present analysis

| Abbreviation | Population | Perfecture |
|---|---|---|
| Okn | Okinoerabu | Kagoshima |
| Tng | Tanegashima | Kagoshima |
| Ksk | Kushikino | Kagoshima |
| Tmk | Tomioka | Kumamoto |
| Fke | Fukue | Nagasaki |
| Imj | Imajuku | Fukuoka |
| Ngt | Nagato | Yamaguchi |
| Nim | Nima | Shimane |
| Dai | Daiei | Tottori |
| Hms | Hamasaka | Hyogo |
| Hkt | Hikatae | Toyama |
| Kkz | Kakizaki | Niigata |
| Mjm | Majima | Niigata |
| Atm | Atsumionsen | Yamagata |
| Nkh | Nikaho | Akita |
| Iws | Iwasaki | Aomori |
| Kss | Kasose | Aomori |
| Srh | Shirahama | Miyazaki |
| Nth | Natahama | Ooita |
| Mrt | Muroto | Kochi |
| Mhm | Mihama | Wakayama |
| Irk | Irako | Aichi |
| Int | Inatori | Shizuoka |
| Cts | Chitose | Chiba |

*DNA isolation and polymerase chain reaction (PCR)*

From about 300 mg of young leaf, total DNA was isolated by the method of Dellaporta et al. (1983), with slight modifications, and used as a template for a subsequent PCR. Figure 1 shows the three synthesized oligonucleotides and the target regions for amplification employed in the present analysis. The oligonucleotides were synthesized in an automated DNA synthesizer (Model 391A, Applied Biosystems Inc., USA) following the manufacturer's instructions. Primers A, B and C correspond to *trnfM*, *ORF105* and *atp6*, respectively. The nucleotide sequences are taken from Makaroff et al. (1989).

PCR was performed using the primer pairs A and C (no. 1), and B and C (no. 2). PCR with primers A and C should amplify a 331-bp DNA fragment in radishes with normal mtDNA. On the other hand, PCR using primers B and C should yield both 546- and 474-bp DNA fragments in a plant with Ogura-type mtDNA, since the target sequence for primer B exists in duplication in *ORF105* (Fig. 1). Thirty PCR cycles were performed in a programmable incubator (Zymoreactor, Atto Co. Ltd., Japan). Each cycle consisted of denaturation for 1 min at 94 °C, annealing for 2 min at 55 °C, and extension for 3 min at 72 °C. The 50-μl reaction mixture contained 25 pmol of each primer and 1.0 unit of Tth DNA polymerase (Toyobo Co. Ltd., Japan) in the manufacturer's buffer system. After PCR, a DNA fragment was separated by electrophoresis using a 4% Nusieve (FMC BioProducts, USA) agarose gel.

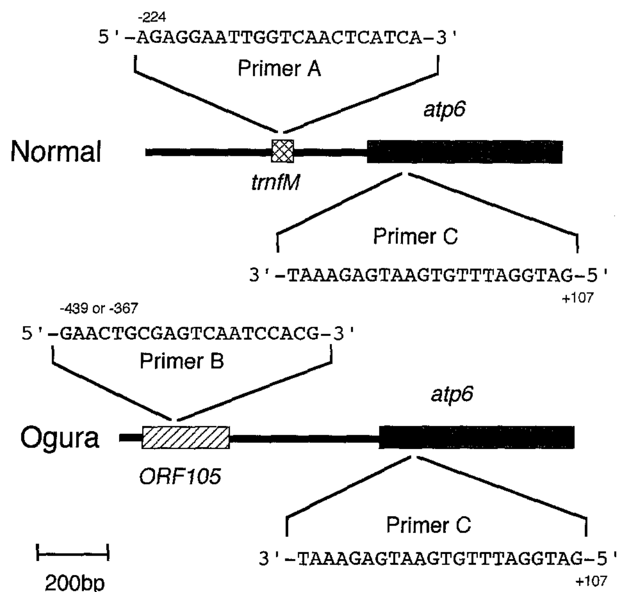


**Fig. 1.** Oligonucleotides used as PCR primers to amplify the upstream regions of the mitochondrial *atp6* gene. The nucleotides are numbered from the predicted translation start site of an *atp6* gene (Makaroff et al. 1989)

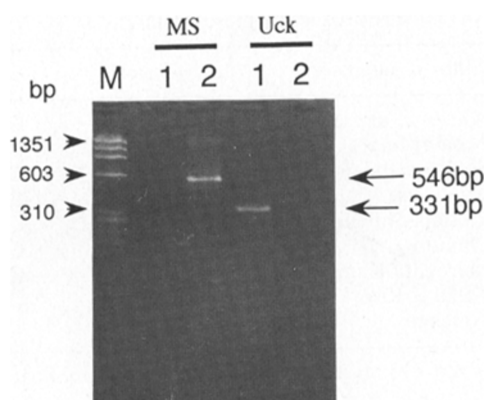


**Fig. 2.** Amplification products after PCR using the total DNA from MS-Gensuke (*MS*) and Uchiki-Gensuke (*Uck*) as a template. *Numbers 1 and 2* indicate PCR no. 1 using primers A and C, and PCR no. 2 using primers B and C (see Fig. 1). *M*: $\phi$X174 *Hae*III digest used as a molecular size standard

## Results

### *Distinction between normal and Ogura-type mtDNAs by PCR*

Figure 2 shows the amplification products of 'Uchiki-Gensuke' and 'MS-Gensuke' after the two PCR procedures. In 'Uchiki-Gensuke', which is a maintainer of Ogura male-sterile cytoplasm, a 331-bp DNA fragment of normal mtDNA was amplified by PCR no. 1, and no DNA fragment was amplified by PCR no. 2. On the other hand, 'MS-Gensuke' showed a 546-bp fragment of Ogura mtDNA after PCR no. 2, whereas no product appeared after PCR no. 1. Although another 474-bp fragment in Ogura mtDNA should have appeared with PCR no. 2, it was not observed under our experimental conditions. However, these results demonstrated that the normal and Ogura mtDNA were distinguishable by the combined PCR.

### *Mitochondrial type of cultivated radishes*

All the cultivars analyzed here (Table 1) had a DNA band specific to the normal mtDNA, while that expected in Ogura mtDNA was not observed (data not shown). Four individual plants were analyzed per cultivar, and there were no variations in the band positions within each. Thus, the mtDNAs of cultivars belonging to the main Japanese radish cultivar groups were regarded as the normal type.

### *Distribution of Ogura-type mtDNA in wild radish*

Figure 3 shows examples of amplification products of wild radish from the selected populations after the PCR, whereas Fig. 4 summarizes mtDNA variations among and within wild radish populations. As shown in these figures, both normal and Ogura-type mtDNAs are present in wild Japanese radishes. As described

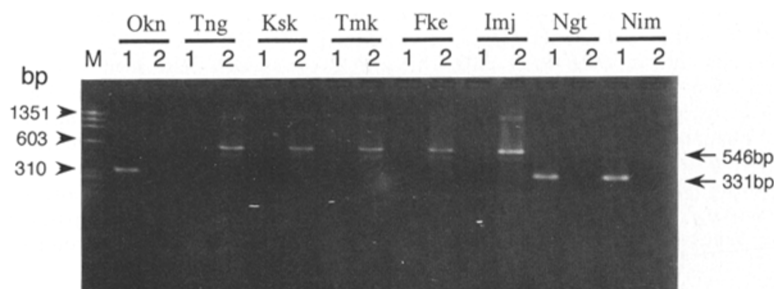


**Fig. 3.** Examples of amplification products of Japanese wild radishes after PCRs nos. 1 and 2. The abbreviation above the lane shows the population from which the wild radishes were collected (Table 2). *M*: $\phi$X174 *Hae*III digest used as a molecular size standard

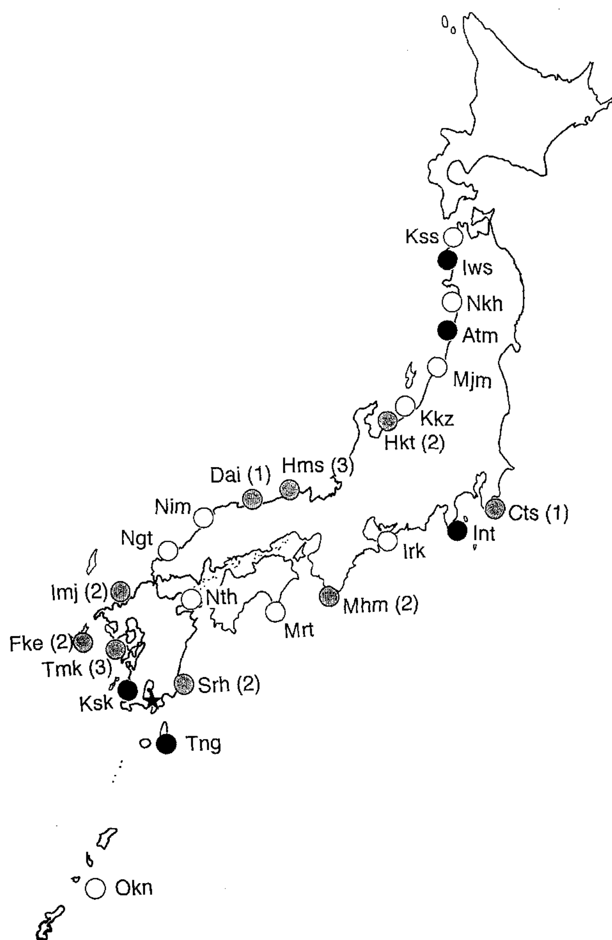


**Fig. 4.** The distribution and frequency of normal and Ogura-type mtDNAs in Japanese wild radishes. *White circles* indicate the populations in which all four plants had normal mtDNA; *black circles* represent those with only Ogura-type mtDNA. *Gray circles* indicate the populations containing both types of mtDNA, and *numerals in parentheses* show the number having Ogura-type mtDNA among the four plants analyzed

earlier, the original Ogura cytoplasm was found in the Kagoshima prefecture (shown as a star in Fig. 4). All four plants in the two populations near this site (Ksk and Tng) show an Ogura-specific amplification pattern. However, Ogura-type mtDNA was not restricted to these areas, and was widely distributed in the other 12 populations. Furthermore, no geographic cline was observed in the distribution pattern.

With regard to the variation of mtDNA type, the 24 populations analyzed here were classified into three groups. Among ten populations, all four plants had normal mtDNA, whereas five populations showed only the Ogura-type mtDNA. The other nine populations included both normal and Ogura-type mtDNAs in each. These results are the first demonstration that the mtDNAs of Japanese wild radishes are differentiated both among allopatric populations and among plants growing sympatrically.

## Discussion

### *Possible origin of Ogura male-sterile cytoplasm*

In Japan, wild radishes mainly grow along the coast, and are called 'Hamadaikon', which means 'beach radish'. These wild radishes belong taxonomically to *R. sativus*, and there is no reproductive isolation with the cultivated type despite considerable ecological and morphological differences. We found here that the Ogura-type mtDNA was distributed among the wild radishes collected from various areas of Japan, in contrast to the fact that none of the major cultivars have Ogura-type mtDNA. Both the wild radishes with Ogura-type mtDNA and the cultivar with normal mtDNA have normal pollen fertility, and several major Japanese cultivars lack restorer genes for Ogura male sterility (Ogura 1968).

From these facts we deduced the origin of Ogura male sterility as follows: natural hybridization initially occurred between a female wild radish with Ogura cytoplasm and a male cultivar without restorer genes. Thereafter, in some progenies of this cross, male-sterile mutant segregants were found by Ogura (1968).

### *Possible origin of Ogura-type mtDNA*

The above scenario applies only to phenotypic expression of male sterility; the origin of the Ogura-type mtDNA *per se* remains unresolved. Based on the analysis of the organelle DNAs of normal and Ogura male-sterile radishes, Makaroff and Palmer (1988) argued that Ogura mtDNA was derived from a normal mtDNA of cultivated radish by a series of mutations including the *atp6* region analyzed here. However, we found Ogura-type (mutated) mtDNA in Japanese wild radish populations but not in the radish cultivars, and currently there is no evidence showing the direction of mutation. Although the mutations leading to the differentiation between normal and Ogura-type mtDNA could have occurred in a wild radish, the nature of process involved is still unclear. In the present study, we used only Japanese cultivars and wild radishes grown in Japan and our analysis was limited to the *atp6* gene region of mtDNA. Therefore, to clarify this issue, further analysis of wild material in continental Asia is necessary. It is also necessary to study regions other than *atp6*, since many variations between two mtDNA types have been found (Makaroff and Palmer 1988; Makaroff et al. 1989, 1990, 1991). If a partially-mutated mtDNA could be identified, the evolutionary process of mutation from normal to Ogura-type, or *vice versa*, mtDNA could be reconstructed. These studies

may also allow the allopatric and sympatric mitochondrial differentiation in wild radishes to be resolved.

*The potentiality of wild radish as breeding material*

Although Ogura-type mtDNA can be found in more than ten populations of wild radishes (Fig. 4) they themselves show normal pollen fertility. In order to determine the effect of the mitochondrial genome on the male sterility of radish, we are conducting hybridizations between wild radishes with Ogura-type mtDNA and several radish cultivars. If male sterility is induced in the progenies of the crosses, the wild radishes could be used as breeding material to introduce cytoplasmic male sterility into cultivated radishes and other Cruciferous crops. This would enlarge the range of the genetic diversity among breeding material for Cruciferous crops, since the wild radishes that are widely distributed in Japan contain far larger variations in ecological characteristics than those that are cultivated.

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## References

Bannerot M, Bouliard L, Chupeau Y (1977) Unexpected difficulties met with the radish cytoplasm in *Brassica oleracea.* Eucarpia Cruciferae Newslett 2:16

Bonnet A (1977) Breeding in France of a radish $F_1$ hybrid obtained by use of cytoplasmic male sterility. Eucarpia Cruciferae Newslett 2:5

Dellaporta SJ, Wood J, Hicks JB (1983) A plant DNA minipreparation; version II. Plant Mol Biol Rep 1:19–21

Kao HM, Brown GG, Scoles G, Seguin-Swartz G (1991) Ogura cytoplasmic male sterility and triazine-tolerant *Brassica napus* cv Westar produced by protoplast fusion. Plant Sci 75:63–72

Makaroff CA, Palmer JD (1988) Mitochondrial DNA rearrangements and transcriptional alterations in the male-sterile cytoplasm of Ogura radish. Mol Cell Biol 8:1473–1480

Makaroff CA, Apel IJ, Palmer JD (1989) The *apt6* coding region has been disrupted and a novel reading frame generated in the mitochondrial genome of cytoplasmic male-sterile radish. J Biol Chem 264:11706–11713

Makaroff CA, Apel IJ, Palmer JD (1990) Characterization of radish mitochondrial *atpA*: influence of nuclear background on transcription of *atpA*-associated sequences and relationship with male sterility. Plant Mol Biol 15:735–746

Makaroff CA, Apel IJ, Palmer JD (1991) The role of *coxI*-associated repeated sequences in the plant mitochondrial DNA rearrangements and radish cytoplasmic male sterility. Curr Genet 19:183–190

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 Agr Kagoshima Univ 6:39–78

Pelletier G, Primard C, Vedel F, Chetrit P, Remy R, Rousselle P, Renard M (1983) Intergeneric cytoplasmic hybridization in Cruciferae by protoplast fusion. Mol Gen Genet 186: 453–459

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N (1985) Enzymatic amplifiication of $\beta$-globin genomic sequences and restriction-site analysis for diagnosis of sickle cell anemia. Science 230:1350–1354

Shiga T (1980) Male sterility and cytoplasmic differentiation. In: Tsunoda S, Hinata K, Gómez-Campo C (eds) *Brassica* crops and wild allies. Japan Science Societies Press, Tokyo, pp 205–221