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Distribution of mitochondrial plasmid-like DNA in cultivated rice (*Oryza sativa* L.) and its relationship with varietal groups

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Summary. Mitochondrial (mt) plasmid-like DNA was found in most of more than 100 rice cultivars (*Oryza sativa* L.) by the use of 0.7% agarose gel electrophoresis (AGE). The DNA varied in molecular weight and number. By electron microscopy, small circular DNAs of different sizes could be detected in addition to the DNAs of high molecular weight, even in cultivars in which mt plasmid-like DNA was not detected by AGE. The detection of the mt plasmid-like DNAs by AGE did not depend on their presence or absence, but on their high stoichiometry. The relationship between cytoplasms with mt plasmid-like DNAs and varietal (for example, Indica rice) groups was close. The geographical distribution of cytoplasms is discussed.

Key words: Electron microscopy – Genetic variation – Mitochondrial DNA – *Oryza sativa* L. – Plasmid-like DNA

Introduction

The analysis of mitochondrial (mt) DNA from different plants by agarose gel electrophoresis (AGE) and electron microscopy has shown plasmid-like DNA molecules of low molecular weight that differ in size, stoichiometry, and molecular form; e.g., *Brassica* (Palmer et al. 1983), *Sorghum* (Pring et al. 1982; Chase and Pring 1985), sugarbeet (Powling 1981), *Oenothera* (Brennicke and Blanz 1982), tobacco (Dale et al. 1983), *Zea mays* (Kemble and Bedbrook 1980; Weissinger et al. 1982); *Vicia faba* (Boutry and Briquet 1982; Negruk et al. 1982) *Linum usitatissimum, Datura inoxia, Glycine max, Petunia hybrida,* and *Phaseolus aureus* (Bailey-Serres et al. 1987), rice (Yamaguchi and Kakiuchi 1983), and wheat (Handa et al. 1984).

In maize, the plasmid-like DNAs fall into specific size classes when analyzed by AGE, and the relationship between the plasmid-like DNA and the cytoplasmic characteristics conferring male sterility has been investigated (Kemble et al. 1980). The assay of plasmid-like DNAs has proven to be a simple way to investigate maize cytoplasms (Weissinger et al. 1982). The presence or absence of mt plasmid-like DNAs is independent of nuclear background (Kemble and Bedbrook 1980; Pring et al. 1982), but in some cases the plasmid-like DNA copy number can be affected by the nuclear genotype (Carlson et al. 1982; Erickson et al. 1986; Kemble et al. 1986). An association between mt plasmid-like DNA and the occurrence of cytoplasmic male sterility (cms) has also been suggested in some plants (Levings et al. 1980; Nawa et al. 1987). In rice, differences in mtDNA between maintainer and cms strains (Kadowaki et al. 1986) and among the ten strains with cms (Kadowaki et al. 1988) have been analyzed by restriction endonuclease analysis. Plasmid-like DNAs have been identified by AGE in mitochondria from cms-Bo (Yamaguchi and Kakiuchi 1983) and WA cytoplasm (Mignouna et al. 1987). In seven cms strains, the plasmid-like DNAs were heterogeneous in molecular weight, sequence homology, and molecular form (Kadowaki and Harada, in preparation). This suggests an extensive divergence in plasmid-like DNA, as well as in high molecular weight DNA, the so-called 'master chromosome' of cms rice mitochondria. However, the mtDNAs of rice cultivars have not yet been investigated, and there is, therefore, a lack of information on the homogeneity of their cytoplasms and on the presence or absence of plasmid-like DNAs. The importance of the cytoplasm in regulating vital plant functions such as

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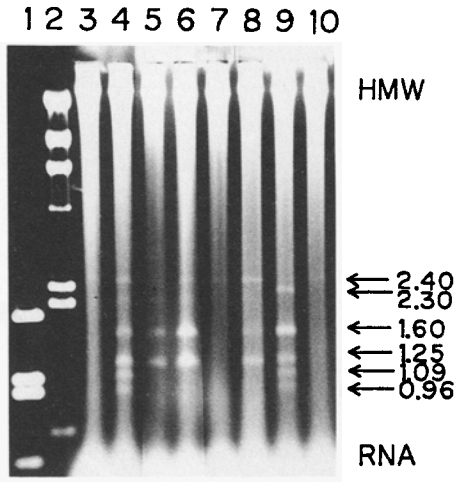

Fig. 1. Distribution and variation of mitochondrial plasmid-like DNAs in rice cultivars. Mitochondrial DNAs isolated from etiolated rice seedlings were incubated with RNase A and separated by electrophoresis on a 0.7% agarose gel without restriction enzyme treatment. *Lane 1,* pBR322 digested with BstNI, as size marker; *lane 2,* lambda DNA digested with HindIII, as size marker; *lane 3,* 'Nan jing xiang dao'; *lane 4,* 'JENA Col. No. 015'; *lane 5,* 'Xuan chang quang'; *lane 6,* 'Dali zaoxian'; *lane 7,* 'Yun 83-132'; *lane 8,* 'Dao ren qiao'; *lane 9,* 'Leulikelash'; *lane 10,* 'China 830'. Varietal groups of the cultivars are as follows: Indica rice, *lanes 4–6* and *8–10*; Japonica rice, *lanes 3* and *7*. The molecular weights (kb) of plasmid-like DNAs are shown in the right margin of the figure. *HMW* and *RNA* refer to high molecular weight mtDNA molecules and digested RNA molecules, respectively

photosynthesis, sugar and fatty acid metabolism, ATP production, cms, and disease susceptibility has been well established. Plant mitochondria are generally considered to be maternally inherited (Levings and Pring 1976), so analyses of rice mtDNA will, by classifying rice cultivars with respect to their cytoplasm, provide useful information for both breeding programs and for the study of phylogenetic and evolutionary relationships in cultivated rice.

In this paper, we report the results obtained when plasmid-like DNA in 102 lines of rice cultivars was analyzed by AGE. This method was used for its simplicity in detecting the molecular basis of mtDNA divergence; restriction endonuclease analysis, on the other hand, gives complex fragment patterns. This is the first report on the diversity of plasmid-like DNAs in cultivated rice mitochondria, and on the relationship between banding patterns of plasmid-like DNA and the varietal groups of cultivated rice.

Materials and methods

Plant materials

For electrophoretic analysis of mtDNA, 102 lines of rice cultivars from collections of genetic stocks provided by the Laboratory of Plant Germplasm Introduction, National Institute of Agrobiological Resources, Japan, were used. They were either indigenous or pure lines. The classification of rice cultivars into varietal groups, such as Indica rice, etc., was based on isozyme analysis, the distribution of gametophyte genes, the degree of hybrid seed sterility, and morphological and physiological characters (Nakagahra 1978, 1986). For the electron microscopic analysis of mtDNA molecules, cvs 'Taichung 65' (Japonica) and 'Auslaljira' (Indica) were used.

Preparation of mtDNA and agarose gel electrophoretic analysis

Rice mtDNA was extracted, purified, and analyzed by 0.7% agarose gel electrophoresis using the methods of Kadowaki et al. (1988) without restriction enzyme treatment. About 15 g etiolated leaves was used as the starting material, and the nucleic acids (6–7 μg) obtained were incubated with 0.3 μg/μl RNase A (Sigma) for 120 min at 37 °C and then immediately put on the gel. The presence or absence of plasmid-like DNAs was assessed from photographs, so only samples with an adequate quality of DNA and with highly resolved electrophoretic patterns were used.

Electron microscopy of small circular mtDNAs

The mtDNA molecules isolated were dissolved in 20 m*M Tris*-HCl (pH 8.0) and 2 m*M* EDTA, and spread by the protein monolayer method of Kleinschmidt (1968). Samples were mounted on films of carbon-coated parlodion (Mallinckrodt, St. Louis, MO, USA) and examined under a JEOL 100C electron microscope at 80 kV.

Results

Presence and distribution of plasmid-like DNAs in cultivated rice mitochondria

The mtDNAs were isolated from etiolated seedlings of rice cultivars, incubated with RNase A, and separated by electrophoresis on 0.7% agarose gels. The 102 rice cultivars examined consisted of 69 Indica and 33 Japonica rice lines. The mt plasmid-like DNAs were identified in gels stained with ethidium bromide. Six plasmid-like DNAs having molecular masses of 2.40, 2.30, 1.60, 1.25, 1.09, and 0.96 kb are shown in lanes 4 and 9 of Fig. 1; three plasmid-like DNAs with molecular masses of 2.40, 1.60, and 1.25 kb, in lanes 5, 6, and 8; one plasmid-like DNA of 2.40 kb, in lanes 3, 7, and 10. Of the 102 cultivars examined, 65 had plasmid-like DNAs with differences in their banding patterns, indicating the heterogeneity of mtDNAs in rice cultivars.

We classified the cytoplasms into three types based on the electrophoretic banding patterns of their plasmid-like DNA. However, because of the small quantities of the 2.40 and 2.30 kb plasmid-like DNAs, the determination of their presence or absence was sometimes difficult. Type I cytoplasm had four plasmid-like DNAs of 1.60, 1.25, 1.09, and 0.96 kb; type II cytoplasm had the plasmid-like DNAs of 1.60 and 1.25 kb, but not those of 1.09 or 0.96 kb; type III cytoplasm had either no plasmid-like DNA, or different types of plasmid-like DNA of 1.60, 1.25, 1.09, or 0.96 kb.

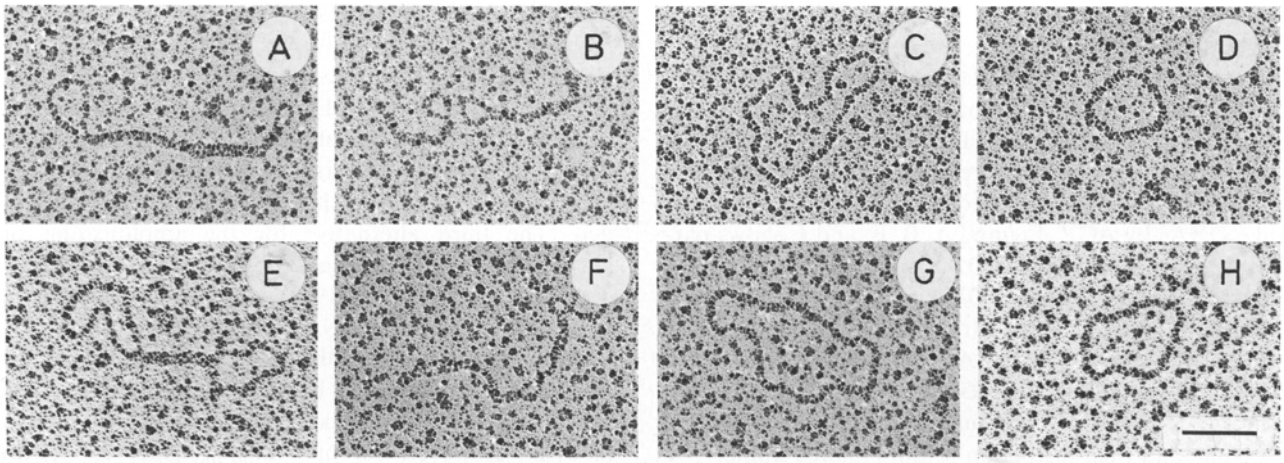

Fig. 2A–H. Small supercoiled and open circular DNA molecules in rice mitochondria observed by electron microscopy. **A–D** DNA molecules from the cultivar 'Taichung 65' (Type III* mt banding pattern). **E–H** DNA molecules from the cultivar 'Auslaljira' (Type I* mt banding pattern). **A, B, E,** and **F** show partially loosened supercoiled DNA molecules, and **C, D, G,** and **H** show open circular DNA molecules. *Bar* = 83 nm. * See Table 1

Table 1. Polymorphism of plasmid-like DNA in Indica and Japonica rice

Banding patterns of mt plasmid-like DNA[a]	Indica	Japonica
Type I	46 (66.7)[b]	1 (3.0)
Type II	13 (18.8)	0 (0)
Type III	10 (14.5)	32 (97.0)
	69	33

[a] Type I cytoplasm has four plasmid-like DNAs of 1.60, 1.25, 1.09, and 0.96 kb
Type II cytoplasm has two plasmid-like DNAs of 1.60 and 1.25 kb, but does not have the 1.09 or 0.96 kb DNAs
Type III cytoplasm has no plasmid-like DNA nor the different plasmid-like DNAs of Type I or II
[b] The numbers in parentheses show percentages in each varietal group

The relationship between the banding patterns of mt plasmid-like DNA and the varietal groups of the rice cultivars was investigated (Table 1). Cytoplasms of Japonica rice were mainly Type III, but the cytoplasms of Indica rice were Types I, II, or III. These results were, in part, in agreement with the findings of Ishii et al. (1986), who showed that the restriction fragment pattern of chloroplast DNA (cpDNA) of the ecospecies (ecosp.) *Indica* is different from those of ecosp. *Japonica* and *Javanica* (included in Japonica rice in a broad sense), and that the restriction patterns of the latter two ecospecies are identical. Our results show even more heterogeneity within the Indica rice cytoplasms, indicating that mtDNA analysis may be more sensitive than cpDNA analysis for investigating the divergence of rice cytoplasms.

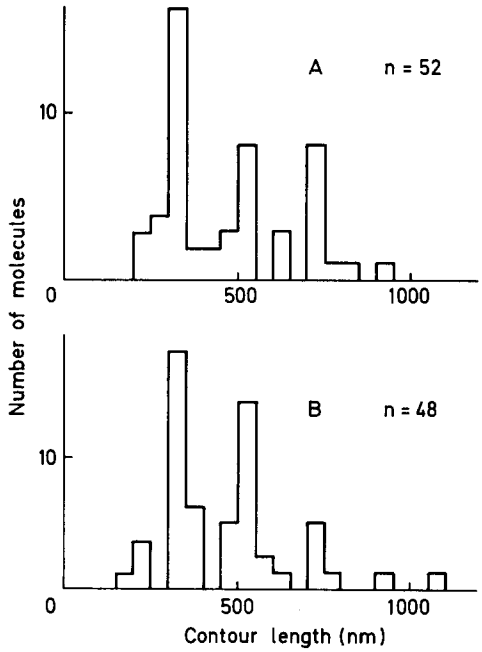

Fig. 3A and B. Histograms of the contour lengths of small circular DNA molecules observed in electron microscope preparations of mtDNAs. **A** DNA molecules from the cultivar 'Auslaljira'; **B** DNA molecules from the cultivar 'Taichung 65'. *n*, number of molecules in each sample

Size distribution of small circular DNA under the electron microscope

MtDNA molecules were prepared from cvs 'Taichung 65' (Japonica), and 'Auslaljira' (Indica), which had Types III and I mt plasmid-like DNA banding patterns, respectively. Electron microscopic observations of rice mtDNA

molecules not only showed small circular DNA molecules, but also huge heterogeneous DNA molecules that were mainly linear (the latter may be preparation artifacts; data not shown). Both small supercoiled DNA molecules and open circular DNA molecules were identified. In both cultivars, the presence of these small circular molecules was independent of the detection of plasmid-like DNAs by AGE. Figure 2A, B, E, and F shows partially loosened supercoiled DNA molecules, and C, D, G, and H show open circular DNA molecules. The contour lengths of the open circular DNA molecules were between 210 and 900 nm in 'Auslaljira' and between 180 and 1100 nm in 'Taichung 65' (Fig. 3). In both cultivars, the modal size of the molecules ranged between 300 and 350 nm, and their histogram profiles looked similar. We also observed circular molecules that might have been the expected replicative intermediates (data not shown).

Discussion

The small circular DNA molecules observed by AGE and electron microscopy in this study may have been the product of intramolecular or intermolecular recombinations in direct or inverted repeat sequences of mtDNA, as proposed for *Brassica campestris* (Palmer and Shields 1984) and maize (Lonsdale et al. 1984). Plasmid-like DNAs like those shown in Fig. 1 should be highly amplified small DNA molecules because of their high molar ratio to the master chromosomal DNA. Regardless of the identification of plasmid-like DNAs by AGE, we found small circular DNAs with similar size distribution profiles in both cultivars (Figs. 2 and 3). The sizes of the 1.60 and 1.25 kb molecules in Fig. 1 have been estimated by Nawa et al. (1987) to actually be 2.2 and 1.5 kb, respectively, because those molecules are circular; while the size of the 1.09 kb molecule is thought to be 1.09 kb because of its linear conformation (Kadowaki and Harada, in preparation). Each of plasmid-like DNAs observed in Fig. 1 seemed to correspond with a peak in Fig. 3 when the length of 1 kb was taken to be 340 nm. Therefore, the detection of plasmid-like DNA by AGE was not due to the existence of small DNA, but to its stoichiometry.

Neither the mechanism nor the factors involved in the high amplification of mt plasmid-like DNA is known. Three possible mechanisms may be involved in the regulation of specific DNA amplification. First, the plasmid-like DNA may be autonomously replicated by its own product. However, this seems unlikely since rice plasmid-like DNAs are very small. Not enough of an open reading frame has been identified to allow coding for a functional protein in the plasmid-like DNA B1 of rice mitochondria (Shikanai et al. 1987), which has an electrophoretic mobility similar to that of the 1.60 kb DNA shown in Fig. 1. Second, amplification of specific DNA may be regulated not by nuclear DNA but by other mtDNA of high molecular weight, as reported by Kemble et al. (1980) and Pring et al. (1982), who obtained characteristic banding patterns of mtDNA regardless of the nuclear background. In this case, plasmid-like DNAs could function as molecular markers for distinguishing between cytoplasms because mitochondria are maternally inherited. The third alternative for the amplification of plasmid-like DNA is regulation by nuclear DNA, as suggested by Erickson et al. (1986) and Kemble et al. (1986), where the plasmid-like DNA copy number is affected by the nuclear genotype. In this case, the gene(s) that regulate amplification might be included among the genes that specify the rice varietal groups, or be closely linked to them. However, our preliminary results do not all agree with this possibility. The plasmid-like DNAs found in Indica rice are present even after recurrent backcrossing to the Japonica rice (Kadowaki and Harada, in preparation), but the effects of specific nucleocytoplasmic interactions on the presence of plasmid-like DNA are still unknown.

Cultivated rice consists of two independent species: *Oryza sativa* L. and *Oryza glaberrima* Steud. Many different approaches have been taken to characterize the cultivars of *O. sativa* into varietal groups. Morinaga (1954) and Chang (1976) classified rice cultivars into the three varietal groups of Indica, Japonica, and Javanica rice on the basis of the degree of hybrid seed fertility after intervarietal crossing experiments. This classification is almost the same as that proposed by Oka (1958), which was based on differences in the physiological and morphological characteristics, and that by Matsuo (1952), based on morphological characteristics. Nakagahra (1978, 1986) further classifies the Indicas into Indica and Sinica (Hsien) rice, according to the conventional methods mentioned above, as well as by analyses of gametophyte genes and isozymes. Glaszmann (1986) classifies the varieties into six groups by analyses of several isozymes. In our experiments, rice cultivars could be classified into three groups by the electrophoretic banding patterns of mt plasmid-like DNA. The relationship between the banding patterns of mt plasmid-like DNA and the four varietal groups, Indica, Sinica, Javanica, and Japonica, of Nakagahra was examined (Table 2). Genetic differences were found in the rice cytoplasms. The great majority of Indica cytoplasms were Type I, the majority of Javanica and Japonica cytoplasms were Type III, and the Sinica cytoplasms were mixtures of Types I, II, and III. Plasmid-like DNAs were identified in all of the cytoplasmically male-sterile rice strains, the cytoplasms of which are from *Oryza rufipogon* (Kadowaki and Harada, in preparation), generally considered to be the ancestor of *O. sativa*. The relationship between the plasmid-like DNA identified in this study and that of *O. rufipogon* is currently being examined.

Table 2. Relationships between banding patterns of mitochondrial plasmid-like DNA and varietal groups of rice[a]

Banding patterns of mt plasmid-like DNA	Indica		Japonica		
	A	B	C	D	
Type I	38 (84.4)	8 (33.3)	0	1 (5.9)	47
Type II	2 (4.4)	11 (45.8)	0	0	13
Type III	5 (11.1)	5 (20.8)	16 (100)	16 (94.1)	42
	45	24	16	17	102

[a] Varietal groups were categorized according to isozyme analysis, distribution of gametophyte genes, degree of hybrid sterility, morphological characteristics, etc. by Nakagahra (1978, 1986)
A: Indica rice collected from India, Sri Lanka, and Bangladesh
B: Sinica (Hsien) rice collected from south China and Vietnam
C: Javanica rice collected from hilly areas of southeastern Asia and tropical islands
D: Japonica rice collected from Japan and northern China

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References

Bailey-Serres J, Leroy P, Jones SS, Wahleithner JA, Wolstenholme DR (1987) Size distributions of circular molecules in plant mitochondrial DNAs. Curr Genet 12:49–53

Boutry M, Briquet M (1982) Mitochondrial modifications associated with the cytoplasmic male sterility in faba beans. Eur J Biochem 127:129–135

Brennicke A, Blanz P (1982) Circular mitochondrial DNA species from *Oenothera* with unique sequences. Mol Gen Genet 187:461–466

Carlson JE, Gabay-Laughnan S, Laughnan JR (1982) Nucleocytoplasmic interactions in *cms*-S of maize. In: Sheridan WF (ed) Maize for biological research. Plant Molecular Biology Assoc; Charlottesville/VA, pp 243–245

Chang TT (1976) The origin, evolution, cultivation, dissemination, and diversification of Asian and African rices. Euphytica 25:425–441

Chase CD, Pring DR (1985) Circular plasmid DNAs from mitochondria of *Sorghum bicolor*. Plant Mol Biol 5:303–311

Dale RMK, Wu M, Kiernan MCC (1983) Analysis of four tobacco mitochondrial DNA size classes. Nucleic Acids Res 11:1673–1685

Erickson L, Grant I, Beversdorf W (1986) Cytoplasmic male sterility in rapeseed (*Brassica napus* L.). 2. The role of a mitochondrial plasmid. Theor Appl Genet 72:151–157

Glaszmann JC (1986) A varietal classification of Asian cultivated rice (*Oryza sativa* L.) based on isozyme polymorphism. In: Rice genetics. IRRI, Manila, The Philippines, pp 83–90

Handa H, Tsunewaki K, Kunisada T, Yamagishi H (1984) Small circular DNA molecules in wheat mitochondria. Mol Gen Genet 194:368–372

Ishii T, Terachi T, Tsunewaki K (1986) Restriction endonuclease analysis of chloroplast DNA from cultivated rice species, *Oryza sativa* and *O. glaberrima*. Jpn J Genet 61:537–541

Kadowaki K, Ishige T, Suzuki S, Harada K, Shinjyo C (1986) Differences in the characteristics of mitochondrial DNA between normal and male sterile cytoplasms of Japonica rice. Jpn J Breed 36:333–339

Kadowaki K, Osumi T, Nemoto H, Harada K, Shinjyo C (1988) Mitochondrial DNA polymorphism in male-sterile cytoplasm of rice. Theor Appl Genet 75:234–236

Kemble RJ, Bedbrook JR (1980) Low molecular weight circular and linear DNA in mitochondria from normal and male-sterile *Zea mays* cytoplasm. Nature 284:565–566

Kemble RJ, Gunn RE, Flavell RB (1980) Classification of normal and male-sterile cytoplasms in maize. II. Electrophoretic analysis of DNA species in mitochondria. Genetics 95: 451–458

Kemble RJ, Carlson JE, Erickson LR, Sernyk JL, Thompson DJ (1986) The *Brassica* mitochondrial DNA plasmid and large RNAs are not exclusively associated with cytoplasmic male sterility. Mol Gen Genet 205:183–185

Kleinschmidt AK (1968) Monolayer techniques in electron microscopy of nucleic acid molecules. In: Grossman L, Moldave K (eds) Methods in enzymology, vol 12B. Academic Press, New York, pp 361–377

Levings CS III, Pring DR (1976) Restriction endonuclease analysis of mitochondrial DNA from normal and Texas cytoplasmic male-sterile maize. Science 193:158–160

Levings CS III, Kim BD, Pring DR, Conde MF, Mans RJ, Laughnan JR, Gabay-Laughnan SJ (1980) Cytoplasmic reversion of cms-S in maize: association with a transpositional event. Science 209:1021–1023

Lonsdale DM, Hodge TP, Fauron CMR (1984) The physical map and organisation of the mitochondrial genome from the fertile cytoplasm of maize. Nucleic Acids Res 12:9249–9261

Matsuo T (1952) Genecological studies on the cultivated rice. Bull Natl Inst Agric Sci 3:1–111

Mignouna H, Virmani SS, Briquet M (1987) Mitochondrial DNA modifications associated with cytoplasmic male sterility in rice. Theor Appl Genet 74:666–669

Morinaga T (1954) Classification of rice varieties on the basis of affinity. In: Studies on rice breeding (a separate volume of the Jpn J Breed, vol 4) pp 1–19

Nakagahra M (1978) The differentiation, classification and genetic diversity of cultivated rice (*Oryza sativa* L.) by isozyme analysis. Trop Agric Res Ser (TARC) 11:77–82

Nakagahra M (1986) Geographic distribution of gametophyte genes in wide crosses of rice cultivars. In: Rice genetics. IRRI, Manila, The Philippines, pp 73–82

Nawa S, Sano Y, Yamada M, Fujii T (1987) Cloning of the plasmids in cytoplasmic male sterile rice and changes of

organization of mitochondrial and nuclear DNA in cytoplasmic reversion. Jpn J Genet 62:301–314
Negruk VI, Cherny DI, Nikiforova ID, Aleksandrov AA, Butenko RG (1982) Isolation and characterization of minicircular DNAs found in mitochondrial fraction of *Vicia faba*. FEBS Lett 142:115–117
Oka HI (1958) Intervarietal variation and classification of cultivated rice. Indian J Genet Plant Breed 18:79–89
Palmer JD, Shields CR (1984) Tripartite structure of the *Brassica campestris* mitochondrial genome. Nature 307:437–440
Palmer JD, Shields CR, Cohen DB, Orton TJ (1983) An unusual mitochondrial DNA plasmid in the genus *Brassica*. Nature 301:725–728
Powling A (1981) Species of small DNA molecules found in mitochondria from sugarbeet with normal and male sterile cytoplasms. Mol Gen Genet 183:82–84
Pring DR, Conde MF, Schertz KF, Levings CS III (1982) Plasmid-like DNAs associated with mitochondria of cytoplasmic male-sterile *Sorghum*. Mol Gen Genet 186:180–184
Shikanai T, Yang ZQ, Yamade Y (1987) Properties of the circular plasmid-like DNA B1 from mitochondria of cytoplasmic male-sterile rice. Plant Cell Physiol 28:1243–1251
Weissinger AK, Timothy DH, Levings CS III, Hu WWL, Goodman MM (1982) Unique plasmid-like mitochondrial DNAs from indigenous maize races of Latin America. Proc Natl Acad Sci USA 79:1–5
Yamaguchi H, Kakiuchi H (1983) Electrophoretic analysis of mitochondrial DNA form normal and male sterile cytoplasms in rice. Jpn J Genet 58:607–611