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Double-stranded RNA and male sterility in rice

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Summary. Double-stranded RNA (dsRNA) was isolated from rice *Oryza sativa* ssp. *japonica,* but not from other subspecies. The dsRNA has been found in all of the examined cytoplasmic male-sterile (CMS) lines of BT (Chinsurah Boro II)-type rice, but was not detected in their companionate maintainer lines. It is uniquely and positivley correlated with the CMS trait in BT-type rice. Recently, the dsRNA was also found in a nuclear male-sterile (NMS) rice, Nongken 58s, but was not found in its normal Nongken 58. The molecular weight of this dsRNA was estimated to be about 18 kb. Electron microscopic analysis reveals that it is linear shapped. The double strandedness of the RNA molecules was characterized by CF-11 cellulose column chromatography and nuclease treatments. It bound to CF-11 cellulose in the presence of 15% ethanol. It was sensitive to RNase A at low salt concentrations, but insensitive to DNase I, S1 nuclease, and RNase A at high salt concentrations. The dsRNA was detected in both mitochondrial and cytoplasmic fractions. Dot-blot hybridization reveals that there is no sequence homology between this dsRNA and mtDNA, but there is homology between this dsRNA and nuclear genomic DNA. We have not been able to transmit this dsRNA to fertile rice.

Key words: Double-stranded RNA – Male sterility – Rice

Introduction

DsRNAs have been detected in fungi and yeast for a long time. Their presence was reported to be associated with the virulent function of some plant pathogens (Koltin and Day 1976; Rogers et al. 1987; Newton et al. 1985; Kim and Klassen 1989). The differences in size and number of the dsRNA segments have even been used as molecular markers to delimit the taxonomic position of several rust fungi (Newton et al. 1985). In recent years, dsRNAs were found in some higher plants, such as maize (Sisco et al. 1984; Finnegan et al. 1986), French bean (Wakarchuk and Hamilton 1985), broad bean (Grill and Garger 1981; Turpen et al. 1988), cassava (Gabriel et al. 1987), tobacco (Ikegami and Fraenkel-Conrat 1979), and rice (Wang et al. 1988; Wang et al. 1989b; Zhang and Wang 1990). Most of these are agronomically important food crops. In some cases the dsRNA was associated with CMS lines. During the study of rice mitochondrial DNA (mtDNA), we found that a special RNA always co-purified with mtDNA. We have reported simply the discovery of dsRNA in rice previously (Wang et al. 1988; Zhang and Wang 1990). In an attempt to learn more of its properties, function, cellular location, and relation to male sterility, we began studying the dsRNA in more detail. Here we report the results obtained.

Materials and methods

Plant materials

Seeds of rice (*Oryza sativa* ssp. *japonica*) Qiu Guang A (CMS line) and B (maintainer line), Xiu Ling A and B, Li Ming A and B were provided by Prof. Hong Lifang (Crop Institute, Beijing Agricultural Academy of Sciences); Nong Hu 26 A and B were provided by Prof. Dong Yanyu (Hunan Agricultural Academy). All of the rice mentioned above has BT-type cytoplasm. Other rice seeds with non-BT type cytoplasms were provided by Prof. Chen Wanxi (The Agricultural Research Institute of Neijang City, Sichuan Province). Photoperiod-sensitive genic male-sterile rice, Nongken 58s, and its normal Nongken 58 were provided by Prof. Lu Xinggui (Food Crop Institute, Hubei Academy of Agricultural Sciences). All of the rice seeds were reproduced in our Experimental Farm Station.

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Purification and characterization of nucleic acid preparations

Rice mtDNA was isolated from 7-day-old dark-grown seedlings by the procedure of Chase (Chase and Pring 1986). The total mitochondrial nucleic acid (mtNA) was also isolated with the same method but not digested with RNase. All of the NA preparations were dissolved in TE buffer except when otherwise instructed and stored at 4 °C. Samples dissolved in TE buffer were treated with DNase 1 (10 μg/ml) or RNase A (5 μg/ml, preheated at 95 °C for 10 min) for 30 min at 37 °C, and then extracted with a phenol chloroform series as usual. Agarose gel electrophoresis was performed on 1% or 0.8% agarose gel as described previously (Wang et al. 1989a). CF-11 cellulose column chromatograph was carried out according to method 2 reported by Morris (Morris and Dodds 1979). Electron microscopy was carried out according to the method of Kleinschmidt (Kleinschmidt 1968). EcoRI-restricted total nuclear DNA or mtDNA was denatured and dot-blotted onto nitrocellulose paper, then hybridized with ^{32}P-labelled dsRNA probe at 65 °C according to Maniatis et al. (1982). The radioactive probe for the hybridization study was prepared from electrophoretically purified dsRNA by ^{32}P end-labelling techniques on the formamide-cleaved RNA molecules (Negruk 1980).

Results

DsRNA in CMS and NMS rice

During the study of rice mtDNA, a special RNA band was found in the agarose gel electrophoretic pattern of CMS line Qiu Guang A, of BT-type rice, but not in its companionate maintainer line, Qiu Guang B. The result is shown in Fig. 1 A. Later, the RNA in this band proved to be a dsRNA, and was detected in three other CMS lines of BT type, but not in their corresponding maintainer lines. Up to the present, this dsRNA has been detected in all of the four detected CMS lines of BT-type rice, but not in their maintainer lines (Table 1). Recently, the dsRNA was detected in an NMS rice, Nongken 58s, but not in its normal Nongken 58. The result is shown in Fig. 1 B.

Electron microscopy and molecular weight determination

On agarose gel electrophoresis as shown in Fig. 1, the dsRNA migrated in front of the major mtDNA band and faster than the 21.8-kb marker. Determining the molecular weight of the dsRNA was difficult because of its large size and the lack of adequate high-molecular-weight dsRNA markers. In order to determine the molecular weight of the dsRNA more precisely and to know its configuration, we examined the dsRNA preparation under the Hitachi H-500 electron microscope. As shown in Fig. 2, the RNA molecules were found to be linearly formed and double-stranded. The average length of the dsRNA molecules was measured to be about 6 μm. Considering that 1 μm is equal to 3 kb, the total molecular weight was calculated to be 18 kb. Taking into account the results from electron microscopy and electrophoresis, we estimated that the molecular weight of the dsRNA was about 18 kb.

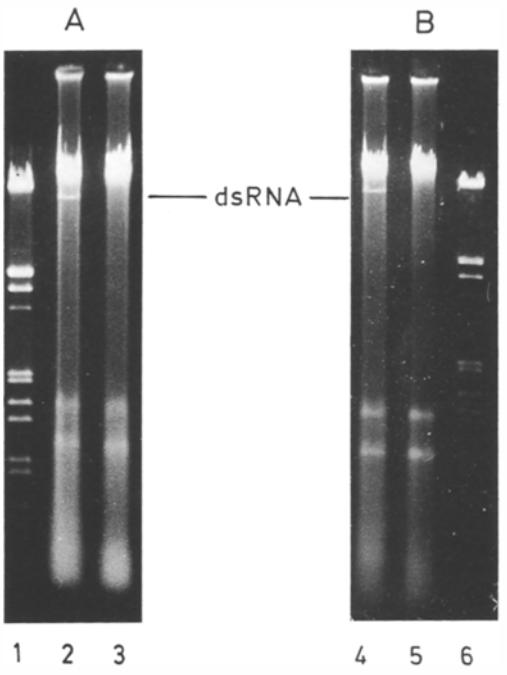

Fig. 1 A and B. The electrophoretic pattern of rice mtNA. *Lanes 1* and *6* – lambda DNA digested with EcoRI and HindIII; *lane 2* – Qiu Guang A; *lane 3* – Qiu Guang B; *lane 4* – Nongken 58s; *lane 5* – Nongken 58

Table 1. The detection of dsRNA in rice

Rice line	Subspecies	Cytoplasm	DsRNA
CMS line			
Qiu Guang A	japonica	BT	+
Qiu Guang B	japonica	BT	–
Xiu Ling A	japonica	BT	+
Xiu Ling B	japonica	BT	–
Li Ming A	japonica	BT	+
Li Ming B	japonica	BT	–
Nong Hu 26 A	japonica	BT	+
Nong Hu 26 B	japonica	BT	–
NMS line			
Nongken 58s	japonica	BT	+
Nongken 58	japonica	BT	–

Characterization of the dsRNA

Electron microscopy showed that the RNA molecules were double-stranded. For further characterization, the total mtNA was treated with DNase 1, RNase A, or S1 nuclease. The results are shown in Fig. 3. The dsRNA was digested by RNase A (lane 4, Fig. 3), but not by DNase 1 (lane 3, Fig. 3) or S1 nuclease (lane 5, Fig. 3). Because dsRNA can specifically bind onto cellulose CT-11

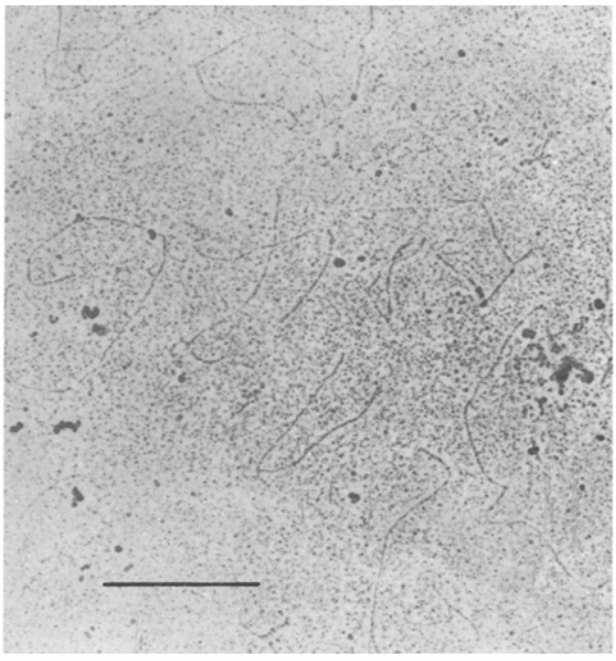

Fig. 2. Electron microscopy of dsRNA molecules. The *bar* represents 3 μm

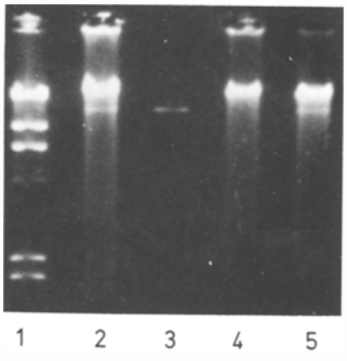

Fig. 3. Nuclease treatments of mtNA. *Lane 1* – lambda DNA digested with HindIII; *lane 2* – mtNA from Qiu Guang A; *lane 3* – 2+DNase 1; *lane 4* – 2+RNase A under low salt conditions; *lane 5* – 2+S1 nuclease

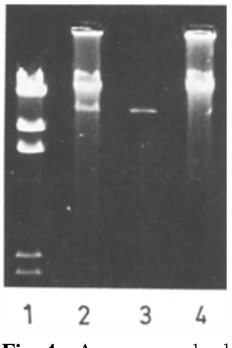

Fig. 4. Agarose gel electrophoresis of mtNA preparations. *Lane 1* – lambda DNA digested with HindIII; *lane 2* – mtNA from Qiu Guang A; *lane 3* – CF-11 cellulose column bound fraction; *lane 4* – CF-11 cellulose column unbound fraction

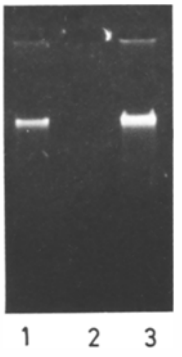

Fig. 5. RNase A analysis of dsRNA purified by CF-11 cellulose column chromatography. *Lane 1* – untreated dsRNA; *lane 2* – dsRNA treated with RNase A at low salt; *lane 3* – dsRNA treated with RNase A at high salt

and Bio-Rad Cellex 410 in the presence of 15% ethanol, we fractionated the total mtNA by CF-11 cellulose column chromatography. The dsRNA bound onto the CF-11 cellulose column in the presence of 15% ethanol. The unbound fraction (effluent void volume) was mainly composed of mtDNA (lane 4, Fig. 4). The bound fraction (elute) contained dsRNA but no mtDNA (lane 3, Fig. 4); it was considered to be purified dsRNA. Because the salt concentration in the eluate was 100 m*M* NaCl, we adjusted the salt concentration to 50 or 300 m*M* NaCl, respectively, in order to determine the sensitivity of dsRNA to RNase A at low salt (50 m*M* NaCl) or high salt (300 m*M* NaCl) concentrations. The result in Fig. 5 indicates that the dsRNA was sensitive to RNase A digestion at low salt concentrations (lane 2, Fig. 5) but resistant at high salt concentrations (lane 3, Fig. 5).

Cellular location of the dsRNA

In order to determine the cellular location of the dsRNA, several cellular fractions from the mtDNA isolation procedure were analyzed on agarose gel electrophoresis. The dsRNA was detected in both the mitochondrial fraction (12,000-g sediment) and the supernatant fraction from which mitochondria had been sedimented. (The supernatant was centrifuged at 45,000-g for 30 min, and dsRNA was isolated from the 45,000-g sediment.) Results are shown in Fig. 6.

DNA-RNA dot-blot hybridization

RNase A-treated rice nuclear DNA and mtDNA were digested with EcoRI, then spotted onto nitrocellulose paper, and hybridized with ^{32}P end-labeled dsRNA probe. As shown in Fig. 7, the dsRNA probe hybridized with nuclear DNA (lane 1, Fig. 7), but not with mtDNA (lane 2, Fig. 7).

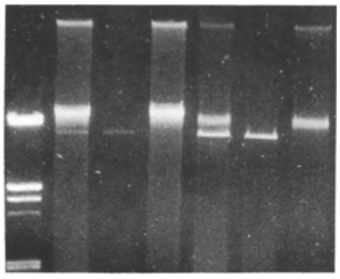

Fig. 6. The identification of rice dsRNA. *Lane 1* – lambda DNA digested with EcoRI; *lane 2* – mtNA prepared by centrifugation at 12,000 -*g*; *lane 3* – 2 + DNase 1; *lane 4* – 2 + RNase A under low salt conditions; *lane 5* – NA prepared from the supernatant of 12,000-g centrifugation (the supernatant was centrifuged at 45,000 -*g*, NA was isolated from the 45,000-*g* sediment); *lane 6* – 5 + DNase 1; *lane 7* – 5 + RNase A under low salt conditions

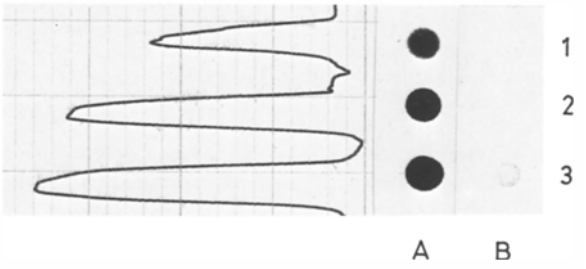

Fig. 7. Dot-blot hybridization between ^{32}P-labeled dsRNA and nuclear DNA and mtDNA. *Lane A* – nuclear DNA; *lane B* – mtDNA. Left part is the scanning of lane A. Dots 1, 2, and 3 each contain 2, 4, and 6 μg DNA equivalent

Discussion

Rice is one of the most important food crops in the world. Male sterilities (including CMS and NMS) are very important characters for hybrid rice production, and are now widely used in China, Japan, and other Asian countries. However, little is known about the molecular mechanism of male sterility. Some scientists found mtDNA was responsible for CMS in rice just as in other crops (Yamaguchi and Kakiuchi 1983; Kadowaki et al. 1986; Kadowaki and Harada 1989; Wang et al. 1987). Some scientists found photoperiod-sensitive genic male sterility was controlled by nuclear genes (Shi 1985; Shi and Deng 1986). For many years, plant breeders speculated that the CMS trait might be caused by virus infection, but there was little evidence, especially at the molecular level. In recent years, CMS-associated dsRNAs found in corn and broad bean support the virus infection theory. An interesting aspect of this study is the discovery of a high-molecular-weight dsRNA in male-sterile rice. It was found in all of the four examined CMS lines of BT-type rice, but not in their companionate maintainer lines. The dsRNA was also found in a nuclear male-sterile line, Nongken 58s, but not in its normal Nongken 58. All of the rice lines in which dsRNA was detected belong to the *japonica* subspecies and have BT cytoplasm. It appears that the presence of the dsRNA is associated with male sterility (including CMS and NMS) of *japonica* rice.

The main point of this paper is the characterization of the dsRNA. Electron microscopy revealed that the RNA molecules were linear shapped. Its molecular weight was estimated to be about 18 kb according to the results from electron microscopy and agarose gel electrophoresis. This agrees with Wang's results (Wang et al. 1989 b). It was a little bit larger than the dsRNAs found in broad bean (16.7 kb) and French bean (14.9 kb and 13 kb), but much larger than that from corn (2.6 and 0.76 kb). Because the RNA is resistant to DNase 1, S1 nuclease, and RNase A under high salt conditions, but sensitive to RNase A under low salt conditions, we speculate that it is double-stranded. The results from CF-11 cellulose column chromatography and electron microscopy confirm that the RNA molecules are double-stranded. As to the cellular location and origin of the dsRNA, this is still not clear. In our experiments, the dsRNA was not only found in mitochondria but also in cytoplasm. Attempts to transmit the dsRNA to fertile rice lines and make them sterile have not been successful. Therefore, we think the dsRNA might not come from viable virus-like particles. The hybridization results showed that there was sequence homology between this dsRNA and nuclear DNA, but not mtDNA. Similar results were observed in French bean, corn, and broad bean. Based on the above results, we speculate that this dsRNA may be derived from an integrated nuclear DNA fragment. The integrated fragment may originally have come from virus during the evolution history. The discovery of male-sterility-associated dsRNA in rice is very interesting for the study on the mechanism of male sterility in rice. However, a lot of work still needs to be done before a conclusion that dsRNA is responsible for male sterility in rice can be drawn.

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