



RNA Isolation from Purified *Rice Necrosis Mosaic Virus* Particles

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Authors' contributions

This work was carried out in collaboration among all authors. Author WSG designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors PMB, DAA and WHB managed the analyses of the study. Author BSA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Rice, particularly grows in tropical and subtropical areas, is one of the most important crops worldwide. *Rice necrosis mosaic virus* (RNMV) was first reported in Japan and causes necrotic lesions on leaf and yellowing. This virus has been classified as member of the Potyviridae family's *Bymovirus* genus. The fungus, *Polymixa graminis*, transmits RNMV. In this study, the methodology first explained RNMV virus particle - purification by ultra-centrifugation along with sucrose and cesium chloride gradient is described. Further, confirmation of purified RNMV particles under the electron-microscope is discussed. In the last RNMV RNA isolation from the purified RNMV particles were explained.

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1. INTRODUCTION

Rice mosaic necrosis virus (RNMV) belongs to the genus *Bymovirus* transmitted by oomycetes, *Polymixa graminis*. RNMV was first reported in rice in Japan and then in India, with symptoms of mosaic in rice characterized by yellow spots and lower leaf streaks [1,2,3,4]. In *Potyviridae* family, the *bymovirus* genus consists of two RNAs, plus-sense RNA virus particles with RNA1 7.3–7.6 kb and RNA2 3.5–3.7 kb [5]. The RNMV is a flexible, rod-shaped RNA-enclosed protein (CP) particle that forms two lengths of filamentous virions: 550 and 275 nm in length and 13 nm in diameter [1]. Both RNAs are covalently linked to the 5' end of the 3' end of the viral genome-linked protein (VPg) and polyadenylated [5]. There are six known species in the *Bymovirus* genus: RNMV, *Barley Yellow Mosaic Virus* (BaYMV), *Barley Yellow Mosaic Virus* (BaYMV), *Wheat Spindle Streak Mosaic Virus* (WSSMV), *Oat Mosaic Virus* (OMV) and *Wheat Yellow Mosaic Virus* (WYMV) [5,6,7,8,9]. Previously Wagh et al. [10], published a complete genome sequence of RNMV-RNA1 and RNA2 and its comparison with *Bymoviruses*.

The rice plants (*Oryza sativa* L. cv. Akebono) were infected by growing them in naturally infested soil with *Polymixa graminis* carrying RNMV from Okayama prefecture in Japan. The virus inoculum used for successful inoculation was RNMV Ka-1 (derived from late Dr. S. Kashiwazaki) [4]. The RNMV inoculation was carried out by sowing the surface sterilized rice (cv. Akebono) seeds on *Polymixa graminis* carrying RNMV-infested soil for 30 days. Further procedures were adopted from Wagh et al [11]. The young seedlings (30 DAS) were placed in growth chambers up to 60 days under control growth conditions. Leaf samples were harvested from 60 days old rice seedlings and used for virus purification. The virus was purified as described previously using CsCl_2 centrifugation [7]. The virus suspension was used for viral RNA extraction. Viral-RNA was prepared as described previously by Moyer and Cali [12], except repetition of Ether steps.

2. MATERIALS AND METHODS

2.1 RNMV Infection to Rice Plant and Confirmation by RT-PCR

Rice cultivar susceptible to RNMV named as Akebono used as a plant material in this study.

Rice plants, supplied by Dr. C. Masuta and Late Dr. S. Kashiwazaki [4], were grown in soil infested with *polymixa graminis* fungal vector. A mixture of two commercial soil materials (Iseki Co. Ltd., Matsuyama, Japan) was filled into small plastic boxes to multiply it with the infected part of the soil. For RNMV inoculation, 1 week old rice seedlings were subsequently transferred from 1/2 MS agar to inoculated soil and placed in the growth chamber with 16/8 hrs light/dark conditions for 2 weeks. The rice seedlings were further transplanted to large plastic pots containing the same soil. The RNMV infected small leaves of four-week-old rice plants (4-leaf stage) were used for total RNA extraction followed by RT-PCR analysis (Fig. 1A) for confirmation of the RNMV infection. The symptoms were observed on the lower leaves after 25-30 days of inoculation as an indication of successful infection (Fig. 1B).

2.2 RNMV Virus Purification by the Sucrose Concentration Gradient Centrifugation

1. Two month old infected leaves were used as virus purification materials. Approximately 12 g of infected leaves were cleaned and ground into 40 mL of 0.1 M citrate buffer with 1/5 volume addition of carbon tetrachloride.
2. Then the crushed sap filtered through the cheesecloth and transferred to 250 mL centrifuge tubes placed on the ice. These tubes were centrifuged at 7000 rpm for 15 min. using ultra-centrifuge (Hitachi CR20G III).
3. Supernatant collected with pipette and transferred to new 250 mL tubes placed on ice, centrifuged again at 10000 rpm for 15 min, similar to e.g. 10 mL tube was shown in Fig. 2A.
4. Supernatant was transferred to 10 mL ultracentrifuge tubes containing 2 mL sucrose solution (20 %) at the bottom of the tube and 8 mL of supernatant. All centrifuge tubes were weighed using weighing balance (Fig. 2B).
5. All tubes were placed into pre-chilled ultracentrifuge rotor, centrifuged at 32500 rpm for 70 min with 20 min acceleration and 20 min slowing time, in a vacuum chamber conditioned at 4°C.
6. After centrifugation, remove the tubes carefully, decant the supernatant without

- disturbing the pellet, green pellet is visible (Fig. 2C), add 2 mL of fresh 0.1 M citrate buffer and keep in inclined position at 4°C for overnight to dissolve virus particles in to buffer (not shown in picture).
7. Repeat step 4, 5 and 6 with 10% sucrose solution to obtain clearer supernatant and pellet with higher concentration of virus particles and minimal amount of plant debris (Fig. 2D, E).
 8. After repeated centrifugation, the volume of partially purified sample increases approximately by 25 percent. Further centrifuge all the content in ultracentrifuge tubes at 36,000 rpm for 20 hours. As a result, the wider (clear) zone of purified RNMV particles will appear towards the bottom of the tube.
 9. The final pellet was dissolved in 4 mL of 0.1 M citrate buffer overnight, and then distributed in small 0.5 mL tubes.

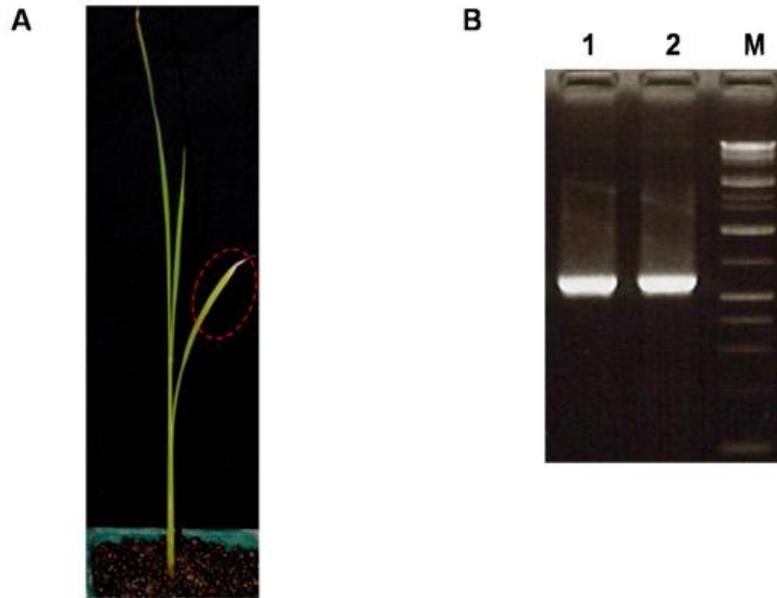


Fig. 1. (A) RNMV symptoms in akebono seedling at early stage and (B) confirmation with RT-PCR, band of 600 bp visualized in two plant samples, the ladder marker is of 10 kb

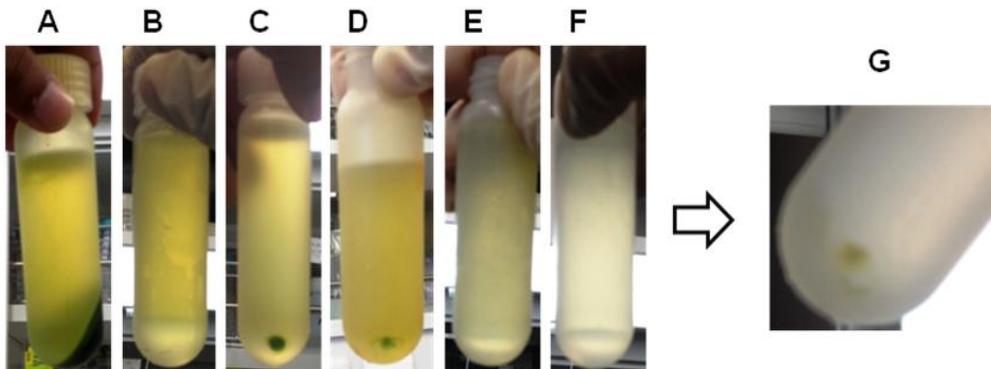


Fig. 2. Viral RNA isolation steps

A. First centrifuge plant sap in citrate buffer; B. Sucrose density gradient in ultra-centrifuge tube; C. Pellet formation after ultracentrifugation; D, E. repetition of Step B and C; F. Cesium chloride density gradient for final centrifugation; G. Purified viral particles as pellet

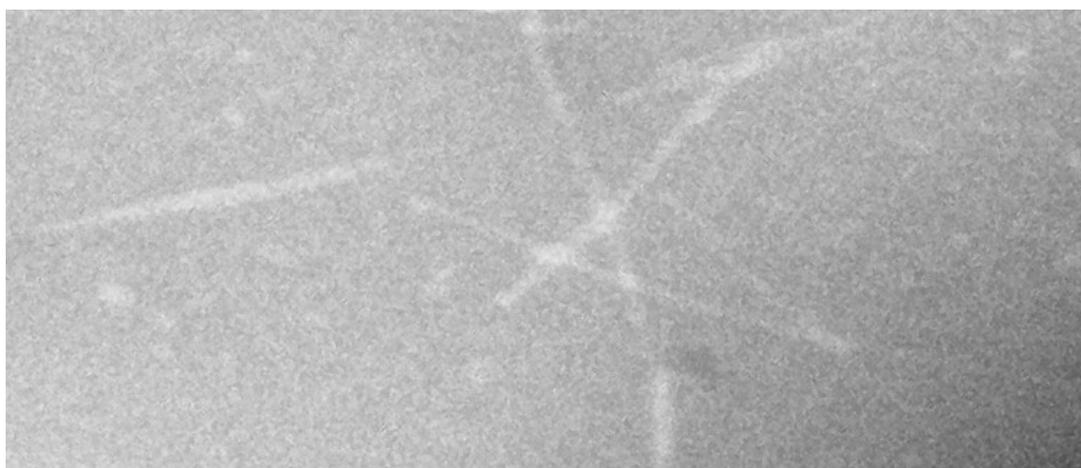


Fig. 3. Rod shaped RNMV virus particles visualized under electron microscope

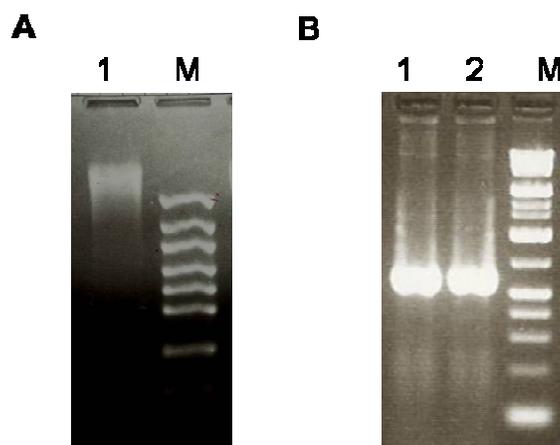


Fig. 4. (A) Purified RNMV RNA, with 5 kb RNA ladder, (B) RT-PCR results of cDNA prepared from isolated RNMV RNA in lane 1 and 2, 600bp band visualized with 10 kb DNA ladder

2.3 Electron Microscopy Visualization of RNMV Particles

To visualize the RNMV particles under the electron microscopy, the purified sample taken into chromic shadows (1 percent final concentration). We diluted 1 mL by 0.1 M citric acid buffer (pH 7.0) solution of 0.2 mL of virus particles and a measured absorbance by 260 nm after measurement of in Nano drop. Confirming the virus density, in diluted samples was further used for visualization under electron microscopy. Preparation of mesh and protocol of electron microscopy was followed as explained in protocol provided by Hitach with instrument. Rod shaped RNMV virus particles seen under the electron microscope (Fig. 3).

2.4 RNMV-RNA Isolation from Purified Virus Particles

The viral RNA purification was carried out by following the protocol given by Moyer and Cali [12], with some modifications. For viral-RNA isolation, virus particles with equal volume of purified virus particles were mixed with 0.05 % (w/v) Proteinase K in proteinase K buffer. Incubate the mixture at 25°C for 20 min. Add equal volume of phenol and shake vigorously, centrifuge at 3000 rpm for 5 min at 4°C, take the supernatant in fresh tube and repeat this step for 3 times. To the supernatant add equal volume of Ether and shake, centrifuge at 3000 rpm for 5 min at 4°C, take supernatant in fresh tube and repeat this step for 3 times. Add 1/10 volume in 3 M sodium acetate, 2.5 volume of chilled absolute

ethanol and keep it for 1 hr in -20°C. Centrifuge it at 10000 rpm for 15 min at 4°C, wash the pellet with 70 percent ethanol, dry the pellet and dissolve in TE buffer. Confirm the concentration of purified RNA by Nano-drop and agarose gel electrophoresis on 1 percent agarose gel with TBE buffer (Fig. 4).

3. DISCUSSION

Purification of plant viruses were largely done in late nineties, there are previously described purification protocols for *bymoviruses*, such as *Barley Yellow Mosaic Virus* (BaYMV), *Barley Yellow Mosaic Virus* (BaYMV), *Oat Mosaic Virus* (OMV), *RNMV*, *Wheat Spindle Streak Mosaic Virus* (WSSMV) and *Wheat Yellow Mosaic Virus* (WYMV) [1,5,6,7,8,9]. Here, we have described the methodology for purification of RNMV virus particles and its RNA for further experiments. In laboratory experiments, we have PCR amplified the viral RNA using the primers described in previous study [11]. After successful infection to rice plants, though this virus shows symptoms on lower leaves, we have confirmed at the presence of viral RNA at molecular level (Fig. 1A, B). Further, 2 months old plants leaves were used for virus purification. The RNMV purification was done by method explained previously [7] with little modifications. In this experiment, we observed similar results as explained previously, but the changes in sucrose cushion gradients were made in the protocol of Usugi and Saito [7]. The pellet visualized with some of greenish part, with clear viral particles (Fig. 2A-G), similar to previous report [8,9]. Virus particles were confirmed as rod shaped, under the electron microscope (Fig. 3). RNMV-RNA was isolated for first time with method given by Moyer and Cali, 1987. RNMV RNA successfully extracted confirmed with Nano drop and agarose gel electrophoresis (Fig. 4A). However, the gel electrophoretic RNA has no comparative image available from *bymoviruses*. These RNA were further converted into cDNA and confirmed by RT-PCR (Fig. 4B). In addition, these RNA were used for sequencing purpose of RNA1 and RNA2 of RNMV in other published study [10]. Finally, this methodology can be further utilized for purification of virus and RNA from virus particles, for experimentation. cDNA prepared by using the RNA isolated from the RNMV or any other viruses by adopting this methodology can then be used for the further bioinformatical analysis. ORF detected can then be easily and cheaply be cloned according to the previously described methodology [13], expressed and characterized.

Advanced technologies like CRISPR/cas [14,15], pathogen interaction with host through small RNA and RNAi mechanism [16,17,18] can also then be employed for further characterization of individual viral proteins both *in vitro* and *in vivo*.

4. CONCLUSION

Here we conclude in this study successfully shown the purification and visualization of RNMV virus particle by cesium sucrose cushion gradient and electron microscope, respectively. Also, the RNMV RNA isolation from the purified virus particle.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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