RNA Isolation from Purified Rice Necrosis Mosaic Virus Particles

Sopan Ganpatrao Wagh\textsuperscript{1,2*}, Manoj Baliram Pohare\textsuperscript{1}, Abhijit Arun Daspute\textsuperscript{3}, Hanumant Baburao Wadekar\textsuperscript{1} and Sachin Ashok Bhor\textsuperscript{1}

\textsuperscript{1}The United Graduate School of Agricultural Sciences, Ehime University, Matsuyama, Ehime 790-8566, Japan.
\textsuperscript{2}Department of Bio-science and Technology, Agricultural Biotechnology College, VNMK University, Aurangabad 431003, MS, India.
\textsuperscript{3}Laboratory of Plant Cell Technology, Faculty of Applied Biological Sciences, Gifu University, Gifu 501-1193, Japan.

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.

Article Information

DOI:10.9734/ACRI/2019/v19i3

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.

*Corresponding author: Email: swagh.gene@gmail.com;
Keywords: Rice necrosis mosaic virus; purification; viral RNA isolation; bymovirus; rice.

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. Kashiwasaki) [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 CsCl2 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. Masutaand Late Dr. S. Kashiwasaki [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.
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 (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 Usugui 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 cecium sucrose cushion gradient and electron microscope, respectively. Also, the RNMV RNA isolation from the purified virus particle.

ACKNOWLEDGEMENT

We thank Dr. Masamichi Nishiguchi for overall guidance and help in virus particle and RNMV RNA purification. We are grateful to Meander Kumar Gakula for checking the English in the manuscript. This research work was supported in part by the Program for the Promotion of Basic and Applied Research in Bio-oriented Industry and Science, the Technology Research Promotion Program for Agriculture, Forestry, Fisheries and Food Industry, the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant-in-Aid for Scientific Research for Scientific Research (C), No. 24580065) to MN by grants from the Ministry of Agriculture, Forestry and Fisheries of Japan (Rice Genome Project to MN) and the Student Research Project Creation Support Program 2015-16 from the United Graduate School of Agricultural Sciences (UGAS), Ehime University, Matsuyama, Japan to SGW.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Inouye T, Fuji S. Rice necrosis mosaic virus. CMI/AAB Descriptions of Plant Viruses, No. 1977;172.
2. Fuji S. Studies on rice necrosis mosaic virus. Special Bull Okayama Pref Agri Expt Sta. 1978;69:81.
3. Ghosh SK. Rice necrosis mosaic. Proc, Indian Acad Sci, Plant Sci. 1980;89:291-299.
4. Badge JL, Kashiwazaki S, Lock S, Foster GD. A bymovirus PCR primer and partial
nucleotide sequence provides further evidence for the recognition of *rice necrosis mosaic virus* as a *bymovirus*. European J Plant Pathol. 1997;103:721-724.

5. Adams M, Zerbini F, French R, Rabenstein F, Stenger D and Valkonen J. Family Potyviridae. Virus Taxonomy, 9th Report of the International Committee for Taxonomy of Viruses. Elsevier Academic Press, San Diego, USA. 2012;1069–1089.

6. Kashiwazaki S. The complete nucleotide sequence and genome organization of barley mild mosaic virus (Na1 strain). Arch Virol. 1996;141:2077-2089.

7. Usugi T, Saito Y. Purification and serological properties of barley yellow mosaic virus and wheat yellow mosaic virus. Ann Phytopathol Soc Japan. 1976;42:12–20.

8. Usugi T, Kashiwazaki S, Omura T and Tsuchizaki T. Some properties of nucleic acids and coat proteins of soil-borne filamentous viruses. Ann Phytopathol Soc Japan. 1989;55:26-31.

9. Zheng T, Chen J, Chen JP, Adams MJ. The complete sequence of *Oat mosaic virus* and evidence for deletion and duplication in RNA2. Arch Virol. 2002;147:635–642.

10. Wagh SG, Alam MM, Kobayashi K, Yaeno T, Yamaoka N, Toriba T, Hirano HY, Nishiguchi M. Analysis of rice RNA-dependent RNA polymerase 6 (OsRDR6) gene in response to viral, bacterial and fungal pathogens. J Gen Plant Pathol. 2016a;82:12-17.

11. Wagh SG, Kobayashi K, Yaeno T, Yamaoka N, Masuta C, Nishiguchi M. *Rice necrosis mosaic virus*, a fungal transmitted *Bymovirus*: complete nucleotide sequence of the genomic RNAs and subgrouping of *bymoviruses*. J Gen Plant Pathol. 2016b;82:38-43.

12. Moyer JW, Calli BB. Properties of sweet potato feathery mottle virus RNA and capsid protein. J Gen Virol. 1987;66:1185-1189.

13. Pohare MB, Akita M. A Rapid and Simple, Recombination-Based Cloning Method in *Escherichia coli*. Biosci, Biotechnol Res Asia. 2017;14(1): 43-52. Available: http://dx.doi.org/10.13005/bbra/2415

14. Pohare MB, Sharma M, Wagh SG. CRISPR/Cas9 genome editing and its medical potential. In Kumar S (Ed.), Advances in Biotechnology and Biosciences. 2019;69-90.

15. Wagh SG, Pohare MB. Current and future prospects of plant breeding with CRISPR/Cas. Current Journal of Applied Science and Technology. 2019;38(3):1-17. Available:https://doi.org/10.9734/cjast/2019/v38i30360

16. Wagh SG. Comparative updates on host-induced gene silencing and CRISPR- Cas9 Utilization for Improved Disease Resistance in Crops, Research Journal of Biotechnology; 2019.

17. Dandve MS, Wagh SG, Bhagat PR, Pawar K, Timake SA, Daspute A A, Pohare MB. Bacterial and fungal pathogen synergetics after co-infection in the wheat (*Triticum aestivum L*.) Biotechnology Journal International; 2019; 23(4): 1-9.

18. Wagh SG, Alam MM, Kobayashi K, Yaeno T, Yamaoka N, Toriba T, Hirano HY, Nishiguchi M. Relationship between resistance to *Rice necrosis mosaic virus* and expression levels of rice RNA dependent RNA polymerase 6 (OsRDR6) in various rice cultivars, JARQ; 2019.