Molecular detection of watermelon mosaic virus associated with a serious mosaic disease on *Cucurbita pepo* L. in Shanxi, China

Yanbing Niu¹ · Xiaojing Pang¹ · Yuanyuan Cheng¹ · Defu Wang¹ · Shang Guo² · Yong Liu³

Received: 22 January 2017 / Accepted: 20 June 2017 / Published online: 29 June 2017
© Indian Virological Society 2017

Abstract With continued expansion of *Cucurbita pepo* L. cultivation, viral diseases affecting the crop have become more serious in recent years, causing enormous losses in yield and quality. A virus sample was obtained from Wenshui in Shanxi province, China. Double-stranded RNA technology and sequence-independent amplification (SIA) were used to identify the virus that induced *C. pepo* L. mosaic disease. SIA and sequencing results showed the presence of watermelon mosaic virus (WMV) in diseased *C. pepo* L. leaves. The complete sequence of WMV from the Shanxi isolate (i.e., WMV-WS) was cloned and analyzed for further characterization. The genomic RNA of WMV-WS is 10,040 nucleotides in length and encodes a putative polyprotein of 3218 amino acids. Phylogenetic analysis indicate that all WMV isolates were divided into four groups and WMV-WS isolate belong to Group 4. Further analysis showed that these WMV isolates were not only to a certain degree related to the host, but also related to geographical origin of isolates. Our results provide

information for a better understanding of the genetic diversity of WMV isolates infecting *C. pepo* L. in Shanxi, China.

Keywords *Cucurbita pepo* L. · Watermelon mosaic virus · Pathogen identification · Analysis of sequences

Introduction

Cucurbita pepo L. belongs to the family Cucurbitaceae and is widely consumed worldwide as a source of bioactive food compounds [24], which possesses various pharmacological properties [20, 25, 26] including an antidiabetic role [3, 8, 17], anti-ulcer role, analgesic, nephro-protection [9], and anticancer effects [25]. However, as *C. pepo* L. cultivation expands, viral diseases have also become a threat for sustainable production of cucurbits [15, 23]. Virus-infected plants commonly appear dwarfed with yellow or light green mottling, leaf deformation, and marginal yellowing [2]. A previous study indicated that *C. pepo* L. is mainly infected by watermelon mosaic virus (WMV), zucchini yellow mosaic virus (ZYMV), and cucumber mosaic virus (CMV) [7, 10]. WMV is one of the economically important plant viruses in the world and has a wide range of hosts, including at least 170 plant species from 27 plant families. WMV can infect all tissues and causes adverse effects in cucurbits [6]. Our investigation about field disease revealed that a majority of *C. pepo* L. exhibited typical mosaic symptoms in Shanxi, which seriously affected their yield and quality.

At present, many detection methods have been established for viral diseases, such as biology detection, electron microscope, ELISA [4], RT-PCR [18], LAMP [14],

Electronic supplementary material The online version of this article (doi:10.1007/s13337-017-0388-2) contains supplementary material, which is available to authorized users.

✉ Yanbing Niu
niuyanbingbest@163.com

¹ College of Life Sciences, Shanxi Agricultural University, No.01. Mingxian Road, Taigu 030801, Shanxi Province, China

² Institute of Edible-Fungi, Shanxi Academy of Agricultural Sciences, Taiyuan 030006, China

³ Key Laboratory of Pest Management of Horticultural Crop of Hunan Province, Hunan Plant Protection Institute, Hunan Academy of Agricultural Science, Changsha 410125, China

et al. However, these techniques are useless for unknown viruses because they dependent on morphology, antigenic or sequence specificity of viruses. Double-stranded RNA (dsRNA) molecules are formed during virus replication, and are comprised of full-length copies of the genomic and subgenomic RNAs viruses. When viruses infect plants, dsRNA as a replication intermediate can detect only from virus-infected tissue and never isolated from healthy tissue [12, 22]. Sequence-independent amplification (SIA) as a technique to identify viral nucleic acid is based on a random PCR ideally leading to representative amplification of all viral nucleotide sequences templates entered into the reaction, which can detect unknown viruses with high sensitivity [19]. Therefore, the viruses infect in plant can be quickly verified by combining dsRNA and SIA.

In this study, leaves of *C. pepo* L. with characteristic symptoms of curling and yellowing were found in fields from Wenshui in Shanxi Province, China. DsRNA technology and SIA were used to identify the viruses that induced *C. pepo* L. mosaic disease. Results showed that the *C. pepo* L. was infected by WMV. The complete genome sequence of WMV-Wenshui isolate from Shanxi, China (i.e., WMV-WS) was cloned and analyzed. The results of this may provide a better understanding of the genetic diversity of WMV isolates and can help in designing better virus control strategies.

Materials and methods

Isolation of dsRNA

Leaves of *C. pepo* L. exhibiting large chlorotic mosaic virus infection were collected from Wenshui in Shanxi Province, China in 2014 (Fig. 1a). The samples were stored at -80°C for subsequent testing.

The viral dsRNA was isolated using CF-11 cellulose powder according to the method described previously by Niu et al. [13] and Tzanetakis et al. [21]. Briefly, approximately 5 g of sample was pulverized in liquid nitrogen. Then, 10 mL of extraction buffer (20 mM NaCl, 10 mM Tris-HCl pH 8.5, 20 mM EDTA, 1.25% SDS, 2% polyvinylpyrrolidone, 1% β -mercaptoethanol) was added, and then the mixture was centrifuged at $3600\times g$ for 10 min at 4°C . The supernatant was collected and an equal volume of phenol-chloroform (25:24) was added to remove proteins. CF-11 cellulose powder was used to isolate dsRNA. 3 M sodium acetate (600 μL) was added to precipitate the dsRNA and the dsRNA pellet was washed with 3 mL 75% ethanol and centrifuged at 4°C for 15 min at $12,000\times g$, then air-dried at room temperature and dissolved in 50 μL of distilled water.

Fig. 1 Field symptoms and PCR detection of *C. pepo* L. infecting samples. **a** Field symptoms of *C. pepo* L. infected by viruses. **b** Electrophoresis of SIA products. M: DL 2000TM DNA marker; Lane 1–3: Infected plant; CK: Healthy plant

Sequence independent amplification (SIA) of WMV-WS genome

To determine whether the virus in the leaf samples remained infectious, first-strand cDNAs from the dsRNA extracts were generated through reverse-transcription polymerase chain reaction (RT-PCR) using M-MLV reverse transcriptase (TaKaRa, Japan) and random reverse primer XTN269 [1] according to the manufacturer's instructions.

SIA was performed in 25 μL reaction mixtures containing 2.0 μL of cDNA, 18.75 μL of DEPC-treated water, 2.5 μL of $10\times$ PCR Buffer, 1.0 μL of primer XTN177, 0.5 μL of dNTP mix (10 mM), and 0.25 μL of Taqplus DNA polymerase (Sangon Biotech, Shanghai, China). The PCR conditions were as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 90 s; and a final elongation step at 72°C for 10 min. The PCR products were analyzed by electrophoresis in 1% agarose gel and visualized by ethidium bromide staining.

To further verify that the sample was infected with WMV, eleven specific primer pairs for the complete genome sequence of WMV were designed based on its conserved regions, which were obtained from multiple sequence alignments of the corresponding isolates in GenBank. All the primers used in this study are listed in Supplementary Table S1.

Cloning and sequence analysis

The amplified fragments were extracted from the agarose gel and purified using an agarose gel DNA purification kit (Sangon Biotech, Shanghai, China) by following the

manufacturer's recommendations. The purified fragments were directly cloned into the pGEM-T vector (Promega), and transformed into *Escherichia coli* strain DH5 α . Three recombinant plasmids in every fragment were randomly selected for sequencing by Sanger sequencing. Nucleotide sequence contigs were assembled using the SeqMan tool from the DNASTAR package (DNASTAR, Inc., Madison, WI, USA) and analyzed against other closely related viral gene sequences using the BLAST program at NCBI web-site (<http://blast.Ncbi.Nlm.Nih.Gov/Blast.cgi>). Multiple sequence alignment was performed using optimal alignment by DNAMAN software package (Lynnon, Biosoft, Canada). The phylogenetic tree was established using MEGA version 5.2 with the neighbor-joining method. Bootstrap analysis with 1000 replicates was performed to evaluate the significance of the internal branches.

Results

DsRNA isolation and SIA detection

The symptomatic leaves of *C. pepo* L. produced a distinct dsRNA band of about 10 kb, which was gel purified and used as the template for viral cDNA preparation. A 1500-bp DNA fragment was amplified from symptomatic plants using XTN177 as primer, but no positive PCR product was obtained in healthy samples (Fig. 1b). BLAST analysis showed that these sequences shared 90–94% identities with other reported WMV isolates, which indicated that *C. pepo* L. was probably infected by WMV, and named as WMV-WS.

Complete genome sequencing and analysis of WMV-WS

To further verify the existence of WMV-WS, the complete genome of WMV-WS was amplified with eleven specific primers (Supplementary Table S1) and sequenced from the infected samples. The fragments with expected sizes of 925, 967, 1363, 1017, 1125, 1486, 1070, 918, 1292, 766, and 1247 bp were amplified (Supplementary Fig. S1). The WMV-WS genome consists of 10,040 nucleotides (nt) (accession number KX664483), determined by assembling the 11 amplified fragments using DNASTAR software. Its genomic organization is typical for members of genus *Potyvirus* and is consistent with that of other reported WMV sequences, with a large open reading frame (ORF) encoding a polyprotein. The larger ORF consists of 9657 nt, encoding a putative polyprotein of 3218 amino acids with a predicted molecular weight of 365.9 kDa. The ORF starts at nt position 133 and ends with a stop codon at positions 9787–9789. The 5' and 3' UTRs consist of 132

and 251 nt, respectively. The polyprotein has nine putative cleavage sites and is cleaved into ten mature proteins (P1, HC-Pro, P3, 6K1, CI, 6K2, VPg, NIa, NIb, and CP). The cleavage site in the P1/HC-Pro dipeptide is predicted to be cleaved by the P1 serine proteinase, whereas the cleavage site in the HC-Pro/P3 is cleaved by the HC-Pro cysteine proteinase. In addition, the cleavage sites of the seven other dipeptides are cleaved by the NIa-Pro cysteine proteinase. The predicted cleavage sites are as follows: IQHY₄₄₄/S (P1/HC-Pro), YRVG₉₀₁/G (HC-Pro/P3), VSAQ₁₂₄₈/A (P3/6K1), VKVQ₁₃₀₀/S (6K1/CI), VQLQ₁₉₃₄/S (CI/6K2), VTTQ₁₉₈₇/G (6K2/NIa-VPg), VEVE₂₁₇₇/S (NIa-VPg/NIa-Pro), VAVQ₂₄₂₀/S (NIa-Pro/NIb), and VSLQ₂₉₃₇/S (NIb/CP). These results confirmed that the symptomatic sample was indeed infected by WMV.

Phylogenetic analysis of WMV-WS

Total 85 complete genome sequences of WMV isolates from different areas are retrieved from the NCBI database, including four WMV isolates from China. Phylogenetic analysis based on the complete amino acid sequence of WMV-WS and other WMV isolates showed that WMV isolates were divided into four groups (Fig. 2). For different host from China, a single isolate from *Ailanthus altissima* (accession number AIA65161.1) was in Group 1 shown in purple, a isolate from *Panax ginseng* (accession number APX42423.1) was in Group 2 shown in green and WMV-CHN (accession number ABD59007.1) and WMV-Shaanxi (accession number AGE83528.1) from *Watermelon* were clustered into Group 3 as indicated in blue, while WMV-WS isolate from *C. pepo* L. was in Group 4 shown in red. Analysis of 85 WMV isolates in NCBI demonstrated that there are nine WMV isolates from *C. pepo* L.. For the same host in different regions, we found that a majority of *C. pepo* L. isolates were from France and two isolates from South Korea. Furthermore, all isolates from South Korea and five isolates from France were clustered into Group 3, but another two isolates from France and WMV-WS isolate from China were divided into Group 4. These indicate that WMV isolates were not only to a certain degree related to the host, but also related to geographical origin of isolates.

Discussion

Plant viruses are extremely important pathogens that cause remarkable damage to plant production. More than 90% of the plant viruses are RNA viruses, wherein replication in its host will produce specific double-stranded RNA, which has been used to determine the virus species and genera

Fig. 2 Phylogenetic tree based on complete genome sequence of WMV-WS and other WMV isolates from different parts of the world and crops. Phylogenetic tree was constructed by the NJ method with 1000 bootstrap replicates, and bootstrap support is indicated at branch

according to the size of dsRNA [5, 12, 27]. Additionally, SIA is a rapid amplification method, which can be amplify independently of any sequence requirements of the DNA or any a priori knowledge of sequence characteristics of the DNA to be amplified [11]. In this study, the infected leaves of *C. pepo* L. were identified through a combination of dsRNA and SIA methods. This technology has overcome the limitations of traditional virus detection methods, which can detect a single virus infection, as well as a complex infection of viruses.

To the best of our knowledge, WMV naturally infects watermelon, melon, pumpkin, cucumber, and zucchini among others and poses serious problems in many parts of China, including Shaanxi, Shandong, Yunnan, Liaoning, Shanxi, Xinjiang, and Heilongjiang [16, 28]. In China, the complete

points. The scale bar represents the number of nucleotide replacements per site. The sequence of *soybean mosaic virus* (accession number HQ396725.1) from China was used as a outgroup

genome sequence of WMV from watermelon has been widely reported, such as WMV-CHN and WMV-Shaanxi, which were isolated from field-grown watermelon plants showing typical virus symptoms in Yangling (Shaanxi, China) in 2005 and in 2011 [29], respectively. But there are no reports about the complete genome sequence of WMV on *C. pepo* L. in China. In this study, the complete sequence of a WMV isolate is reported, which was acquired from field-grown *C. pepo* L. plants exhibiting typical chlorotic mosaic virus infection in Wenshui (Shanxi, China) in 2014, and comparison of phylogenetic and diversity analysis of WMV isolates demonstrated that WMV-WS isolate has close evolutionary relationships with other known WMV isolates. However, WMV-WS has a wide evolutionary distance from WMV-CHN and WMV-Shaanxi isolates from China, which divided

into a separate branch, and whether the dominant reason behind this can be attributed to the host or the region is not entirely clear. Therefore, a comprehensive knowledge of the worldwide structure of WMV populations and further analysis of the complete genome sequence are needed to clarify the biology and characteristics of WMV-WS. This study can help our understanding of the genetic diversity of WMV isolates infecting *C. pepo* L. The findings can also be used in designing better virus control strategies and diagnostic tools, as well as in understanding virus epidemiology and evolution.

Acknowledgements This study was financially supported by grants from the special Fund for Agro-scientific Research in the Public Interest (Grant No. 201303028) and National Natural Science Foundation of China (Grant Nos. 31540050 and 31601612).

References

- Agindotan B, Perry KL. Macroarray detection of plant RNA viruses using randomly primed and amplified complementary DNAs from infected plants. *Phytopathology*. 2007;97(1):119–27.
- Aisan G, Nemat SB, Nahid M. Sequencing part of watermelon mosaic virus genome and phylogenetical comparison of 5 isolates with other isolates from world. *J Agric Food Tech*. 2012;2(6):93–101.
- Chandrasekar B, Mukherjee B, Mukherjee SK. Blood sugar lowering potentiality of selected Cucurbitaceae plants of Indian origin. *Indian J Med Res*. 1989;90:300–5.
- Clark MF, Adams AN. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J Gen Virol*. 1977;34(3):475.
- Dodds JA, Morris TJ, Jordan RL. Plant viral double-stranded RNA. *Phytopathology*. 1984;22(22):151–68.
- Elbeshehy EK, Metwali EM, Almaghrabi OA. Antiviral activity of Thuja orientalis extracts against watermelon mosaic virus (WMV) on *Citrullus lanatus*. *Saudi J Biol Sci*. 2015;22(2):211–9.
- Fletcher JD, Wallace AR, Rogers BT. Potyviruses in New Zealand buttercup squash (*Cucurbit maxima* Duch.): yield and quality effects of ZYMV and WMV 2 virus infections. *N Z J Crop Hortic Sci*. 2000;28(1):17–26.
- Huseini HF, Darvishzadeh F, Heshmat R, Jafariazar Z, Raza M, Larijani B. The clinical investigation of *Citrullus colocynthis* (L.) Schrad fruit in treatment of Type II diabetic patients: a randomized, double blind, placebo-controlled clinical trial. *Phytother Res*. 2009;23(8):1186–9.
- Jain A, Singhai AK. Effect of *Momordica dioica* Roxb on gentamicin model of acute renal failure. *Nat Prod Res*. 2009;24(15):1379–89.
- Lecoq H, Dafalla G, Desbiez C, Wipfscheibel C, Delecolle B, Lanina T, et al. Biological and molecular characterization of Moroccan watermelon mosaic virus and potyvirus isolate from eastern Sudan. *Plant Dis*. 2001;85(5):547–52.
- Malboeuf CM, Yang X, Charlebois P, Qu J, Berlin AM, Casali M, et al. Complete viral RNA genome sequencing of ultra-low copy samples by sequence-independent amplification. *Nucleic Acids Res*. 2013;41(1):218–25.
- Morris TJ, Dodds JA. Isolation and analysis of double-stranded RNA from virus-infected plant and fungal tissue. *Phytopathology*. 1979;69(8):854–8.
- Niu YB, Yao M, Wang DF, Wang GJ, Gong Q. Identification of the pathogenic virus on *Ailanthus altissima*. *Acta Phytopathol Sin*. 2011;41(4):437–40.
- Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, et al. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res*. 2000;28(12):E63.
- Nováková S, Flores-Ramírez G, Glasa M, Danchenko M, Fiala R, Skultety L. Partially resistant Cucurbita pepo showed late onset of the Zucchini yellow mosaic virus infection due to rapid activation of defense mechanisms as compared to susceptible cultivar. *Front Plant Sci*. 2015;6:263.
- Purcifull DE, Hiebert E. Serological distinction of watermelon mosaic virus isolates. *Phytopathology*. 1979;69(2):112–6.
- Rashidi AA, Mirhashemi SM, Taghizadeh M, Sarkhail P. Iranian medicinal plants for diabetes mellitus: a systematic review. *Pak J Biol Sci*. 2013;16(9):401–11.
- Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*. 1988;239(4839):487–91.
- Stang A, Korn K, Wildner O, Uberla K. Characterization of virus isolates by particle-associated nucleic acid PCR. *J Clin Microbiol*. 2005;43(2):716–20.
- Talukdar SN, Hossain MN. Phytochemical, phytotherapeutic and pharmacological study of *Momordica dioica*. *Evid Based Complement Alternat Med*. 2014;2014:806082.
- Tzanetakis IE, Martin RR. A new method for extraction of double-stranded RNA from plants. *J Virol Methods*. 2008;149(1):167–70.
- Tzanetakis IE, Keller KE, Martin RR. The use of reverse transcriptase for efficient first- and second-strand cDNA synthesis from single- and double-stranded RNA templates. *J Virol Methods*. 2005;124(1–2):73–7.
- Ullman DE, Cho J, German L. Occurrence and distribution of cucurbit viruses in the Hawaiian Islands. *Plant Dis*. 1991;75(4):367–70.
- Veronezi CM, Jorge N. Bioactive compounds in lipid fractions of pumpkin (*Cucurbita* sp) seeds for use in food. *J Food Sci*. 2012;77(6):653–7.
- Vijayakumar M, Eswaran MB, Ojha SK, Rao C, Rawat AK. Antiulcer activity of hydroalcohol extract of *Momordica dioica* roxb. *Fruit*. *Indian J Pharm Sci*. 2011;73(5):572–7.
- Yasir M, Sultana B, Nigam PS, Owusuapenten R. Antioxidant and genoprotective activity of selected cucurbitaceae seed extracts and LC-ESIMS/MS identification of phenolic components. *Food Chem*. 2016;199:307–13.
- Zhang XT, Wu ZJ, Lin QY, Xie LH. dsRNA isolation method and research of plant virology. *J Yunnan Agric Univ*. 2005;20(4):455–8.
- Zhang JX, Yun-Feng WU, Wang R, Luo ZP. Complete nucleotide sequence of watermelon mosaic virus China isolate. *Chin J Virol*. 2007;23(2):153–6.
- Zhao L, Feng C, Hao X, Sou M, Zhang J, Wang Q, et al. Complete genomic sequence analyses of watermelon mosaic virus isolates from China. *J Plant Pathol*. 2014;96(3):585–8.