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SHORT COMMUNICATION

Molecular characterization of *Apple necrotic mosaic virus* identified in crabapple (*Malus* spp.) tree of China

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Abstract

Apple necrotic mosaic virus (ApNMV) was identified in crabapple trees with mosaic symptoms from Zaozhuang, Shandong Province, China, by reverse transcription polymerase chain reaction (RT-PCR) analysis. The complete nucleotide sequences of one isolate from crabapple (ApNMV-Hai) and two isolates from apple (ApNMV-Hua and -Qu) were determined. The sizes of genomic RNA1, 2 and 3 of the three isolates differed from those of the previously reported isolate ApNMV-P126 from Japanese apple, especially RNA3. Compared with the nucleotide (nt) sequence of RNA3 in isolate P126, those in the Hai and Qu isolates were 7 and 33 nt shorter, respectively, and that of isolate Hua was 7 nt longer. Alignment analyses showed that these differences in size were mainly due to differences in the lengths of the 5′ untranslated region (UTR) and the UTR region between the ORFs encoding the movement protein and the coat protein. In the phylogenetic trees constructed using the full genomic sequences of RNA1, 2 and 3, the isolate Hai clustered into a group with the isolate Qu in the RNA1 tree, but formed an individual branch in the RNA2 and 3 trees. Three recombination events were identified in the nucleotide sequences of RNA1 and 2 among the isolates ApNMV-Hai, -Hua, and -Qu. This is the first report of the full genome sequence of ApNMV in crabapple.

Keywords: crabapple, mosaic symptom, *Apple necrotic mosaic virus*, molecular characterization

1. Introduction

Crabapple (*Malus* spp., family *Rosaceae*), is economically valuable and represents an important ornamental apple germplasm resource. It is widely used for landscaping and as a research material and is valued for its strong abiotic stress resistance (Tian *et al*. 2015; Zhang *et al*. 2016). Crabapples are infected by the same pathogens as apples, *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem grooving virus* (ASGV), and *Apple stem pitting virus* (ASPV). Sometimes, ACLSV, ASGV and ASPV or these virus strains was symptomless in apple varieties, but can cause serious damage to some crabapple varieties in the leaves, fruit or stem, so these crabapples can be used as indicators to detect latent viruses in apples (Gilmer *et al*. 1971; Li *et al*. 2017).

In recent years, researchers have found that *Apple mosaic virus* (ApMV) is not the only pathogen causing apple mosaic disease. *Prunus necrotic ring spot virus* (PNRSV), *Cucumber mosaic virus* (CMV), and *Apple necrotic mosaic virus* (ApNMV) are also associated with mosaic symptoms in

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apple (Hu G J *et al*. 2016; Hu Y *et al*. 2016; Noda *et al*. 2017). ApNMV (genus *Ilarvirus*, family *Bromoviridae*) contains three single-stranded positive-sense RNA segments. RNA1 and 2 encode two non-structural proteins, a methyltransferase/NTP-binding helicase and an RNA polymerase, respectively, while RNA3 encodes a non-structural movement protein (MP) and a coat protein (CP) (Noda *et al*. 2017; Xing *et al*. 2018). ApNMV was identified in apple trees with mosaic disease in Japan that originated from China in 2017. This virus was considered to cause mosaic symptoms similar to those induced by ApMV (Noda *et al*. 2017). In this research, virus detection was used to index the pathogen of mosaic disease in crabapple and complete nucleotide sequence of ApNMV from crabapple of China was determined.

2. Materials and methods

A mosaic disease occurred in a crabapple orchard in Zaozhuang, Shandong Province, China, in 2016. The infected trees showed yellowing of leaves, veins, and the surrounding leaf lamina (Fig. 1). Virus detection was performed using leaves with mosaic symptoms by reverse transcription PCR (RT-PCR) analysis. Total RNA was extracted from apple samples and cDNA synthesis was referenced by Hu *et al*. (2018). ACLSV, ASGV, ASPV, *Apple scar skin viroid* (ASSVd), PNRSV, ApMV and ApNMV were detected in the samples and two primer pairs were used for each apple pathogen (Appendix A). The genomic sequences of RNA1, 2 and 3 were amplified by RT-PCR using specific primers designed from the obtained cDNA sequences (Appendix B). The 5′ and 3′ terminal sequences of the viral RNA were obtained by rapid amplification of cDNA ends (RACE) with SMARTer® RACE 5′/3′ Kit (Clontech Laboratories Inc., Palo Alto, CA, USA) following the manufacturer's instructions. The PCR products were purified, cloned and sequenced. The sequences were compared using DNAMAN 6.0. Phylogenetic trees were constructed using the neighbor-joining method with 1 000 bootstrap replicates using MEGA 5.0 Software.

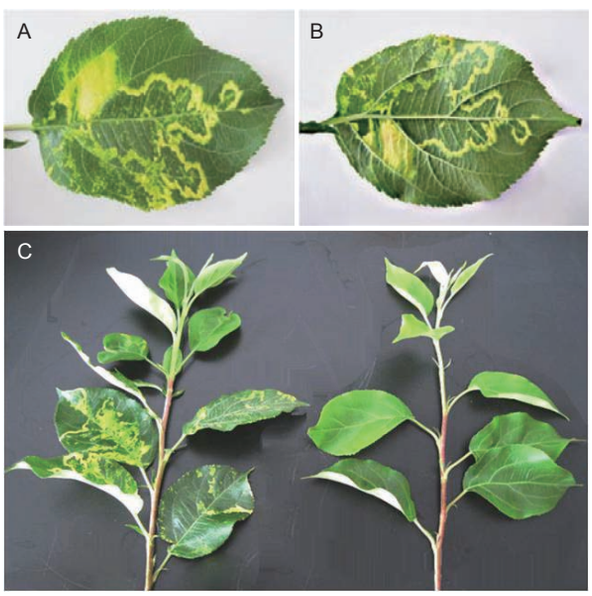

Fig. 1 Mosaic on leaves of crabapple tree with different degrees (A, B and C).

3. Results

After testing, ACLSV, ASGV, ASPV and ApNMV were found in the mosaic symptom crabapple samples. To verify the new ApNMV isolate, full sequences of one crabapple isolate (Hai) and two apple ApNMV isolates (Hua and Qu) were cloned. The lengths of RNA1, 2 and 3 were 3 389, 2 786 and 1 949 nucleotides (nt), respectively, in the isolate Hai (accession nos. MG924894, –897 and –900); 3 387, 2 785 and 1 963 nt, respectively, in the isolate Hua (MG924895, –898 and –901); and 3 387, 2 785 and 1 923 nt, respectively, in the isolate Qu (MG924896, –899 and –902) (Table 1). BLAST searches against the NCBI database identified only one apple ApNMV isolate (P126) with RNA1 (LC108993), RNA2 (LC108994) and RNA3 (LC108995) sequences. The nt identities of RNA1, 2 and 3 of ApNMV-Hai with those of Hua, Qu, and P126 were 94.4–97.5, 93.0–94.6 and 91.1–94.7%, respectively. Similar to the RNA1 of ApNMV-P126 (Noda *et al*. 2017), the genomic RNA1 of the isolate Hai contained open reading frame 1 (ORF1) encoding a putative 120-kDa protein. Its amino acid (aa) identity with the corresponding putative proteins in the Hua, Qu and P129 isolates ranged from 98.5 to 99.5%. RNA2 of the isolate Hai encoded ORF2, which contained a conserved RNA polymerase domain that showed 95.3–96.9% aa identities with those of ORF2 in the other three isolates. The genomic RNA3 of the isolate Hai contained two ORFs, ORF3 and ORF4, encoding an MP and a CP, respectively. The nt and aa sequences of the putative MP of the isolate Hai showed 89.6–95.6% and 91.4–94.6% identity, respectively, with those of Hua, Qu and P129. The CP of the isolate Hai showed higher similarity to that of Qu, with 97.7% identity at both the nt and aa levels. The size of the full genomic sequence of RNA3 differed among the Hai, Hua, Qu and P129 isolates. Compared with the nt sequence of RNA3 in P129, those of Hai and Qu were 7 and 33 nt shorter, respectively, and that of Hua was 7 nt longer (Table 1). The alignment results showed that the lengths of CP and MP of the four isolates were the same, and the differences among isolates were mainly in the 5′ untranslated region (UTR) and the UTR region between the ORFs encoding the MP and CP (Fig. 2-A).

Phylogenetic trees were constructed using the complete

Table 1 Comparison of complete genome sequence and different genomic regions between *Apple necrotic mosaic virus* (ApNMV) isolated from Chinese crabapple (ApNMV-Hai) and other ApNMV isolates (ApNMV-Hua, -Qu and -P129)[1)]

Isolate	RNA1			RNA2			RNA3							
							Genome		MP gene			CP gene		
	nt	nt%	aa%	nt	nt%	aa%	nt	nt%	nt	nt%	aa%	nt	nt%	aa%
ApNMV-Hai	3 389	–	–	2 786	–	–	1 949	–	844	–	–	661	–	–
ApNMV-Hua	3 387	94.4	99.1	2 785	**94.6**	96.5	1 963	93.3	844	**95.6**	**94.6**	661	95.0	94.5
ApNMV-Qu	3 387	**97.5**	**99.5**	2 784	94.3	**96.9**	1 923	94.7	844	91.5	93.6	661	**97.7**	**97.7**
ApNMV-P129	3 378	96.1	98.5	2 767	93.0	95.3	1 956	91.1	844	89.6	91.4	661	93.8	95.4

[1)] nt, nucleotide; aa, amino acid; MP, movement protein; CP, coat protein.
Highest values of nucleotides are indicated in bold.

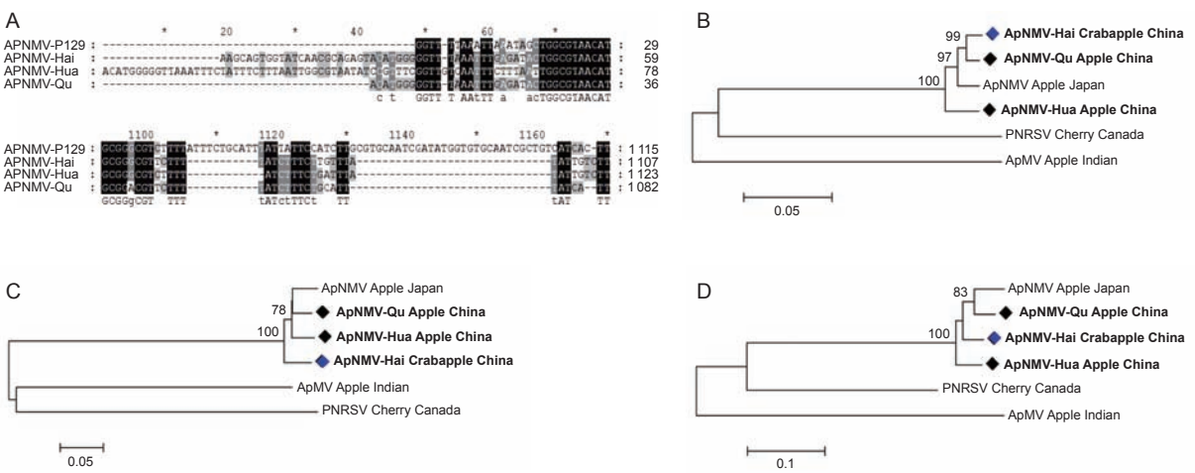

Fig. 2 Alignmentof partial RNA3 nucleotide sequences of *Apple necrotic mosaic virus* (ApNMV) isolates in this study (A). Phylogenetic trees based on full genomicsequences of RNA1 (B), RNA2 (C) and RNA3 (D) from Hai, Hua, and Qu from China and full genomic sequence of ApNMV isolate reported previously. Sources and GenBank accession numbers of reference isolates are listed in Appendix C.

nt sequences of RNA1, 2 and 3 including all ApNMV sequence variants included in this study (Appendix C). The Hai isolate clustered into a group with the Qu isolate in the RNA1 tree, but formed an individual branch in the trees constructed using RNA2 and 3 sequences (Fig. 2-B, C and D). We investigated the occurrence of recombination events, putative recombination junctions, and statistical scores with at least six of the seven programs using the default parameters (highest acceptable probability value=0.05) implemented in the software package RDP4. We identified one recombination event in the RNA1 sequence of Hai, two recombination events in the RNA2 sequences of Hua and Qu, and a potential recombinant in the RNA2 of Qu (Appendix D).

4. Discussion

Apple mosaic disease was the first reported in northeastern United States (Bradford and Joley 1933). The fruit yield of infected trees could decline as much as 30–50%, the growth could reduce 50% and trunk diameter could decrease 20% (Sutic *et al*. 1999). Mosaic symptom could be found in many apple producing areas of China from 1955 and severely threatened the development of the apple industry (Wei 1959; Li *et al*. 2002). As one of the most important rootstocks of apple, many crabapple rootstock cultivars are used in China, e.g., *M*. *robusta* Rehd., *M*. *toringoides* (Rehd) Hughes., *M*. *sieboldii* (Regel) Rehd. and *M*. *prunifolia* Borkh. (Bai *et al*. 2008). The identification of ApNMV in crabapple urgently required us to process the virus detection before graft and use the healthy propagating material to control apple viruses.

The mosaic symptom of crabapple in this study was typical symptom of mosaic disease. However, the pathogen of ApMV could not be found during detection. The same results were found in previously report (Noda *et al*. 2017; Xing *et al*. 2018). ApNMV was considered as the pathogenic factor causing apple mosaic disease in China, so direct biological evidence need to prove the hypothesis. Anessential step in any phylogeny-based analysis is to screen for and quantify evidence of recombination, which is often detected among viruses of the same species, especially among members of the genera *Ilarvirus*, *Cucumovirus* and *Bromovirus*.

The polyphyletic origin of *Ilarvirus* could be the result of recombination events (Shiel and Berger 2000). Three recombination events were found among ApNMV isolates acquired in this study. Although there is no clear evidence for the importance of recombination events in generating these mosaic-causing virus strains, we speculated that such events have contributed to their evolution.

5. Conclusion

In summary, ApNMV was found in crabapple of China showing mosaic symptom. Complete genome sequences of three isolates (ApNMV-Hai, Hua and Qu) were determined from crabapple and apple trees in China. To our knowledge, this is the first report of the full genome sequence of ApNMV from crabapple. These sequences details would form the basis for development of molecular diagnostic and hence a more effective disease control strategy.

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Appendices associated with this paper can be available on http://www.ChinaAgriSci.com/V2/En/appendix.htm

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