Diseases Caused by Viruses

Viral Small RNA-Based Screening of *Malus* spp. Reveals Citrus Leaf Blotch Virus Infection of Kaido Crab Apple in China

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Citrus leaf blotch virus (CLBV, *Citivirus*, *Betaflexiviridae*) is often associated with yellow vein clearing, leaf mottle, and bud union crease symptoms on infected plants. CLBV is reported from many countries, and its host range includes several *Citrus* species and hybrids, as well as Nagami kumquat (*Fortunella margarita* [Lour.] Swingle), peony (*Paeonia lactiflora*), sweet cherry (*Prunus avium* L. cv. Red Lamp), and kiwifruit (*Actinidia chinensis*). CLBV is mainly transmitted by grafting and seed. In June 2018, 60 leaf samples expressing distinctive symptoms including leaf mottle were collected from *Malus* species including apple (*Malus domestica*) and Kaido crab apple (*M. micromalus* Makino) rootstocks, grown from imported and local budwood in an orchard in Tai'an, China. Total RNA was extracted from each sample using CTAB following the method of Su et al. (2016), and then 5 µl of RNA from each sample was mixed together. The total RNA mixture was sent to Beijing Genomics Institute (Shenzhen, China) for sRNA library construction and sequencing using a BGISEQ-500 sequencer. De novo assembly of the total 26,513,625 sRNA reads was performed using Velvet 1.2.10 as described (Su et al. 2016), followed by BLASTn and BLASTx searches against nonredundant nucleotide (nt) and protein databases. Sequences from several viruses known to infect *Malus* spp. were detected, including apple stem grooving virus, apple necrotic mosaic virus, apple geminivirus, and apple hammerhead viroid-like RNA. Additionally, 16 contigs to CLBV were identified, and they represented 14% of the total virus genome sequence. The largest contig (113 nt) was 90% identical to the CLBV HBYD isolate

identified previously from sweet orange (MG572236.1). To further validate the presence of CLBV in *Malus* spp. samples, total RNA from each sample was extracted individually and used in RT-PCR with the primers that span the 5' and 3' termini of the CLBV coat protein (CP) ORF (CP-F, ATGAAAATCACCAATGATAATG; CP-R, CTACATTTC TATGAGTTTTGCCTTG). Amplicons of the expected size (1,092 base pairs) were generated from two crab apple samples, gel purified, and sequenced (MK355658 and MT612990). These two CLBV CP sequences showed 0.8% nt divergence between each other and 88% nt and 94% amino acid (aa) identity to CLBV HBYD isolate (MG572236 and AXG50151). Phylogenetic analysis of CLBV and other *Betaflexiviridae* CP sequences available in GenBank using MEGA7 (Kumar et al. 2016) revealed that the CLBV isolate from crab apple was most closely related to CLBV HBYD isolates and CLBV isolate BT1 (AUY61802.1) (Li et al. 2018). Molecular criteria for species demarcation in *Betaflexiviridae* is that isolates of different species should have <72% nt identity or <80% aa identity. Further, 58 samples from 58 trees including different apple germplasm and wild apple species were randomly collected in the orchards nearby for detection of CLBV by RT-PCR with the same CP primer used in detecting CLBV in crab apple. One sample (cv. Yanhongmi) out of 58 was positive for CLBV. Two symptomatic and two asymptomatic crab apple trees were further tested for the presence of CLBV using primers to amplify the CP gene. RT-PCR results revealed CLBV in these two symptomatic trees, whereas CLBV was not detected in the asymptomatic samples. The CP PCR validation was processed by Sanger sequencing. In addition, a CLBV infectivity trial was also carried out by rub-inoculating *Nicotiana benthamiana* leaves with crude extracts from CLBV-infected crab apple leaves in sodium phosphate buffer (0.2 M). Systemic infection was confirmed by RT-PCR using same primer designed for CLBV CP mentioned above at 3 weeks postinoculation; however, only mild faint mosaic leaves were observed on systemically infected leaves of *N. benthamiana* plants. To our knowledge, this is the first report of CLBV infecting plant species in the *Malus* genus. As a perennial shrub, crab apple may serve as a reservoir for CLBV and poses a risk to other trees in the orchard.

References:

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