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NEW OR UNUSUAL DISEASE REPORTS

Biological and molecular detection of *Hibiscus chlorotic ringspot virus* infecting *Hibiscus rosa-sinensis* in Iran

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Summary. During 2011, ten leaf samples of *Hibiscus rosa-sinensis* with virus-like symptoms including diffuse mottling, numerous chlorotic spots and chlorotic ring spots, were collected from Guilan province, North Iran. The results of mechanical inoculation of sap from these leaves onto different indicator hosts showed local lesions (chlorotic or necrotic) on *Gompherena globosa*, *Chenopodium amaranticolor*, *C. quinoa* and *Gossypium hirsutum,* while *Hibiscus cannabinus* (kenaf) showed systemic chlorotic ring spot symptoms. Purified preparations of the isolated virus contained isometric particles approximately 28–30 nm in diameter. A specific band of about 1.3 kb was amplified from all symptomatic leaves using *Hibiscus chlorotic ringspot virus* (HCRSV) specific primers. Comparative sequence analysis revealed that the IRN.HCRSV (JX865593) isolate shared the greatest nucleotide sequence identity (96.5%) with NC-003608 (Singapore) and the least nt sequence identity (91.5%) with DQ392986 (Taiwan) isolates. The phylogenetic tree showed at least two subgroups for HCRSV isolates in which the Iranian isolate was grouped with Singapore HCSRV (NC-003608). This is the first report of HCRSV in *H. rosa-sinensis* in Iran.

Key words: Chinese hibiscus; Carmovirus; coat protein.

Introduction

Hibiscus rosa-sinensis, commonly known as the Chinese hibiscus, China rose and shoe flower, is an evergreen flowering shrub native to East Asia. It is widely grown as an ornamental plant throughout the tropics and subtropics. Numerous varieties, cultivars, and hybrids are available, with flower colors ranging from white through yellow and orange to scarlet and shades of pink, with both single and double sets of petals (Brunt and Spence, 2000).

Hibiscus chlorotic ringspot virus (HCRSV) was first identified in a hibiscus cultivar imported to the United States from El Salvador (Jones and Behncken, 1980). The virus occurs worldwide in ornamental *H. rosa-sinensis* cultivars (Waterworth *et al*., 1976; Jones and Behncken, 1980). HCRSV is a positive-sense RNA virus with icosahedral particles, and it is a member of the genus *Carmovirus* in the family Tombusviridae (Huang *et al*., 2000; King *et al*., 2012). The genomic RNA of this virus contains four ORFs. A polypeptide of about 28 kDa is encoded by ORF1 and a polypeptide of about 88 kDa (ORF1RT) originates from read-through of the amber terminator of ORF1. Two small polypeptides of about 7 kDa and 9 kDa are coded by ORFs 2 and 3 that are involved in cell-to-cell virus movement. The ORFs 2, 3 and 4 polypeptides are translated from two sgRNAs with sizes of about 1.7 and 1.5 kb. The coat protein (CP) is encoded by ORF4, which is 3′ co-terminal with genomic RNA (King *et al*., 2012).

Symptoms on leaves of HCRSV-infected plants range from generalized mottle to chlorotic ring spots and vein-banding patterns (Waterworth *et al*., 1976). In addition, severe stunting and flower distortion have been observed in hibiscus hybrids grown in the

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tropics (Wong and Chng , 1992). HCRSV is naturally spread by vegetative propagation of infected plants but the virus is not transmitted by seed or the aphid *Myzus persicae* (Brunt and Spence, 2000; Qu and Moris, 2008).

Hibiscus rosa-sinensis is an important ornamental plant frequently used near private and public buildings, in parks and gardens in almost all counties around the Caspian sea, North Iran. Over the last few years, *H. rosa-sinensis* plants showing virus-like symptoms have been observed with incidence reaching up to 50% in some gardens of Guilan province (North Iran). In this paper, we report the isolation and identification of HCRSV in symptomatic *H. rosa-sinensis* and compare the genomic sequence of its CP with those previously published.

Materials and methods

During 2011, ten symptomless and ten symptomatic leaf samples from *H. rosa-sinensis* grown in Guilan province, showing diffuse mottling, numerous chlorotic spots and ring spots, were collected (Figure 1). To verify virus infection, mechanical inoculations were carried out on indicator plants including *Gomphrena globosa* (Amaranthaceae), *Chenopodium quinoa*, *C. amaranticolor* (Chenopodiaceae) and *Alcea rosea, Hibiscus cannabinus* and *Gossypium hirsutum* (Malvaceae). At least six plants of each experimental host were inoculated per each sample. Inoculated *H. cannabinus* (kenaf) (showing necrotic local lesions followed by systemic chlorotic ring spots and yellowing) (Figure 1) was used as a maintenance host for the hibiscus virus, as they source for host range studies and virus purification. Back indexing of inoculated symptomless experimental species was also carried out on kenaf. Fresh *H. cannabinus* leaves from plants inoculated 4 weeks earlier were used for partial purification, using the method of Waterworth *et al.* (1976). For negative staining, purified virus resuspended in phosphate-buffered saline (PBS) was applied to formvar-coated grids and stained with 2% uranyl acetate. Preparations were examined in an EM 300 PHILIPS electron microscope at 80 KV.

Forward Hb-F (5′-GGTT(C/T)TTT(A/G)TCACTGTCCTGAT-3′) (nt 2477-2498) and reverse Hb-R (5′-GGAGGAAAGAAGCAATCAACAT-3′) (nt 3769-3790) primers were designed on the basis of the sequences flanking the CP gene in the complete genome sequence of HCRSV isolates reported in GenBank (Accession No. NC-003608 and DQ392986). Total RNA was extracted from 10 symptomatic samples using QIAGen RNeasy Mini Kit (USA), and first-strand cDNA synthesis was performed using M-MuLV reverse transcriptase (Fermentas, Lithuania), according to the manufacturers' instructions. Polymerase chain reaction (PCR) amplifications were carried out using high fidelity Platinum™ Pfx DNA polymerase (Invitrogen, Carlsbad, CA, USA). PCR products were separated by electrophoresis in agarose gel. The expected fragments were excised from the gels and cleaned by the QIAquick Gel Extraction Kit (QIAGen, Chatsworth, CA USA). DNA sequencing was carried out using the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit and an Applied Biosystems Genetic Analyser DNA model 310 (Applied Biosystems, Foster City, CA, USA). Nucleotide sequence data were assembled using BioEdit Version 5.0.9 (Hall, 1999). The evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1987). *Carnation mottle virus* (CarMV; AF192772) was used as the outgroup to root the tree.

Results and discussion

Mechanical inoculations from symptomatic *H. rosa-sinensis* leaf samples induced local and/or systemic symptoms on different herbaceous species. In particular, *G. globosa* reacted with local reddish lesions (Figure 1). *Chenopodium quinoa* and *C. amaranticolor* reacted with local chlorotic lesions. Both *Ph. vulgaris* and *G. hirsutum* also reacted only locally, and *G. hirsutum* developed necrotic lesions without systemic infection. Necrotic local lesions and systemic chlorotic ring spots were observed on inoculated *A. rosea* (Figure 1). None of the symtomless *H. rosa-sinensis* samples gave reactions on the indicator plants. In electron microscope studies, purified preparations of the isolated virus from infected kenaf seedlings contained isometric particles approximately 28–30 nm in diameter (Waterworth *et al*., 1976; Li and Chang, 2002), whereas no virus-like particles were observed in purified preparations from symptomless leaf samples of *H. rosa-sinensis*.

A product of the expected size (*c.* 1.3 kb) was amplified from RNA extracts obtained from symptomatic samples by RT-PCR. The CP gene sequence of IRN.HCRSV isolate was 1,038 nt, encoding an open reading frame (ORF) of 345 amino acids. Overall nucleotide identity among available HCRSV se-

quences in Genbank ranged between 91.5–100%. However, comparative sequence analysis revealed the maximum (96.5%) nucleotide identities of IRN. HCRSV isolate with NC-003608 (Singapore) and the least nt sequence identity (91.5%) with DQ392986 (Taiwan) isolates. The phylogenetic tree showed at least two subgroups for HCRSV isolates. The Iranian HCRSV was grouped with Singapore isolate (NC-00360) (Figure 2).

HCRSV has been reported from *H. rosa-sinensis* in Australia (Jones and Behncken, 1980), Taiwan (Li and Chang, 2002), El Salvador and USA (Waterworth, 1980). The virus has also been reported from *H. manihot* in Thailand (Jones and Devonshire, 1998), *Hibiscus* sp. in Singapore (Huang *et al*., 2000) and *Abelmoschus manihot* in South Pacific Island countries (Brunt and Spence, 2000). More recently this virus was found in New Zealand (Tang *et al*., 2008). To our knowledge, this is the first report of HCRSV infection in *H. rosa-sinesis* in Iran.

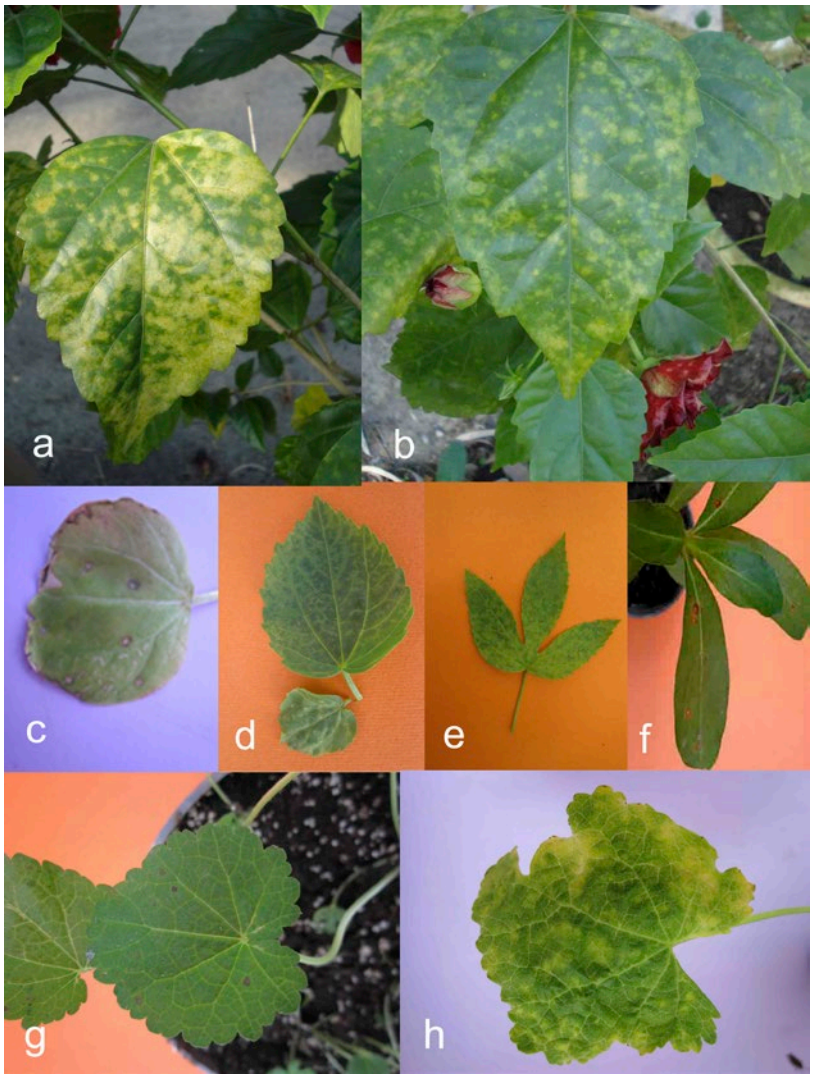

Figure 1. Diffuse mottling (a), chlorotic spots and ring spots (b) in *Hibiscus rosa-sinensis* plants naturally infected by HCRSV. In the host range studies, necrotic local lesions (c) followed by mottling (d), chlorotic spots and ring spots (e) developed in *H. cannabinus*.Necrotic local lesions also developed on *Gomphrena globosa* (f) and *Alcea rosea* (g) followed by chlorotic spots on *A. rosea* (h) after mechanical inoculation with IRN.HCRSV isolate.

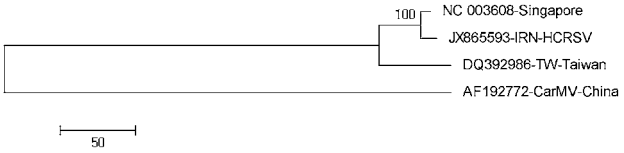

Figure 2. Phylogenetic relationships of the CP nucleotide sequence of the IRN.HCRSV (GenBank JX865593 - 1,014 nt) isolate with four other HCRSV sequences available in Genbank. The tree was constructed by the NJ algorithm implemented by MEGA 5 (Tamura *et al*., 2011). Bootstrap values (1,000 replicates) are given at the branch nodes. *Carnation mottle virus* (AF192772) was used as the outgroup.

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