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Characterisation of Hop stunt viroid (HSVd) isolates from jujube trees (*Ziziphus jujuba)*

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Abstract Hop stunt viroid (HSVd) has been detected in many woody species including grapevine, citrus, peach, plum, apricot, and almond. Here, we report the first detection of HSVd in jujube trees (*Ziziphus jujuba*). Ninety-five samples were collected from jujube trees in Cangzhou, Baoding, and Handan of the Hebei province of China in June 2006. An additional 70 samples were collected from Taigu, the Shanxi province of China in August 2008. From these samples, low molecular weight RNAs were extracted for two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), hybridisation, and reverse transcription polymerase chain reaction (RT-PCR) assays. HSVd was detected in three of the 165 samples, giving an infection rate of 1.8%. The sequences of the HSVd isolates from jujube trees shared 92.6–92.8% homology with the first reported HSVd sequence (GenBank: X00009). Using alignment and phylogenetic analyses, the HSVd sequence variants from jujube were classified in the plum subgroup and were found to be more conserved than other HSVd subgroups.

Keywords HSVd · Jujube · Molecular characterisation · Sequence analysis

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Viroids, which can replicate in host plants and act as phytopathogenic agents, are covalently closed single-stranded RNAs that range in length from 246 to 475 nucleotides. In the viroid genome, there is no coding sequence for a coat protein, in contrast to virus genomes (Diener 1989, 2003; Flores et al. 1997). Viroids are classified into two families (Flores et al. 1998): Pospiviroidae, composed of species with a central conserved region (CCR) devoid of hammerhead ribozymes, and Avsunviroidae, composed of species lacking a CCR yet possessing hammerhead ribozymes performing a self-cleaving function (Flores et al. 2006). In recent years, viroids have been detected in different plants throughout the world, especially in China. Hop stunt viroid (HSVd) is a member of the genus *Hostuviroid*, family Pospiviroidae and has a wide host range that includes herbaceous crops and most stone fruit species. Until now, HSVd has been reported in grapevine, citrus, plum, peach, pear, apricot, cucumber, and almond

plants (Polivka et al. 1996; Shikata 1990; Astruc et al. 1996; Canizares et al. 1999). In this study, we report the detection of HSVd in the jujube tree (*Ziziphus jujuba*) for the first time.

The jujube tree originated in China, the predominant jujube-rich country in the world where the cultivation of the jujube tree has a history of more than four thousand years. The jujube tree has been shown to be infected with phytoplasma (Han 2005). However, until now, there has been no report of any jujube disease associated with a virus or viroid. Since HSVd is known to be distributed worldwide and has already been shown to infect many of the stone fruit species of China (Sano et al. 1989; Yang et al. 2006a, 2006b), we investigated whether HSVd was present in jujube trees. The present work reports the first HSVd isolates identified from jujube trees in China and characterises the HSVd sequences obtained.

Young leaves of 165 jujube trees were collected from four different regions in China (i.e. the Cangzhou, Baoding, Handan of the Hebei province, as well as Taigu of the Shanxi province). Low molecular weight RNAs were extracted according to Li et al. (1995). In brief, 5 g of tissue was dried in liquid nitrogen, extracted with 10 ml of 1 M K_2HPO_4 containing 0.1% β-mercaptoethanol, and homogenised with 10 ml phenol: chloroform (1:1, v/v). After eliminating polysaccharides using 2-methoxyethanol extraction, ethanol precipitation was followed by cetyltrimethyl ammonium bromide (CTAB) precipitation. After the high molecular weight RNAs were removed by LiCl (2M), ethyl alcohol was used to precipitate low molecular weight RNAs which were dissolved in 20 μl distilled water and then separated using 2D-PAGE under non-denaturing and denaturing conditions (Schumacher et al. 1983; Hataya 1999). RNA was transferred to a nylon membrane (Hybond-N^+, Amersham Biosciences) and hybridised with a digoxigenin (DIG)-labelled RNA probe of HSVd prepared using a Dig RNA labelling kit (Roche). Hybridisation was carried out as described in Roche's instruction manual (Roche Diagnostics GmbH, Germany).

Of the 165 jujube samples examined, HSVd hybridisation was positive for three samples. CZ3 and CZ4 were collected from the same orchard in CangZhou of the Hebei province, while TG1 was collected from one of the National Jujube Germplasm Repositories of China in Taigu, Shanxi province. As shown in Fig. 1, only one band was separated by 2D-PAGE and detected using silver staining. A strong signal for hybridisation with the DIG-HSVd RNA probe was detected for this single band (Fig. 1). Three primers specific for HSVd were designed for cloning and sequencing of the three HSVd isolates based on the HSVd sequence with GenBank accession number: D13764. P1 (5'-GCTGGATTCTGAGAAGAGTT-3', complementary to nucleotides 87–106) was used as the primer for reverse transcription (RT). The RT mixture was incubated at 42°C for 60 min, and at 98°C for 5 min. The primer pair P2 and P3(P2, 5'-AACCCGGGGCTCCTTTCTCA-3', complementary to nucleotides 67–84, and P3, 5'-AACCCGGG GCAACTCTTCTC-3', homologous to nucleotides 79–96) were used for polymerase chain reaction (PCR) assays. The RT solution was used as the template of PCR. The PCR parameters consisted of a heat denaturation step at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by a final extension step at 72°C for 7 min.

RT-PCR successfully amplified a full length sequence of HSVd from each of the three samples positive for HSVd according to Northern blot analysis. As shown in Fig. 2, a distinct PCR product of 307 bp was identified with the same size as the positive control PCR product and no product was obtained from the negative control (healthy cucumber) (Fig. 2).

PCR products were purified with a PCR purification kit (TIANGEN), cloned into the pGEM-T vector (Promega), and transformed into *E. coli* DH5α. Selected clones were sequenced using an automated DNA sequencer (ABI PRISMTM 3730XL DNA Analyser) and analysed using DNAMAN Version 5.2.2. In total, 24 independent cDNA clones from the three samples were sequenced. From them, 13 sequence variants were detected and aligned with other HSVd sequences available in the GenBank database using Clustal W (Ver.1.83). In comparison with the first reported HSVd sequence (GenBank: X00009), the sequences of HSVd from jujube shared 92.6–92.8% similarity. A phylogenetic analysis was also performed using the 13 HSVd sequences from jujube and 36 other HSVd sequences previously reported (Pelchat et al. 2003). Using the grouping classification of previous reports, HSVd

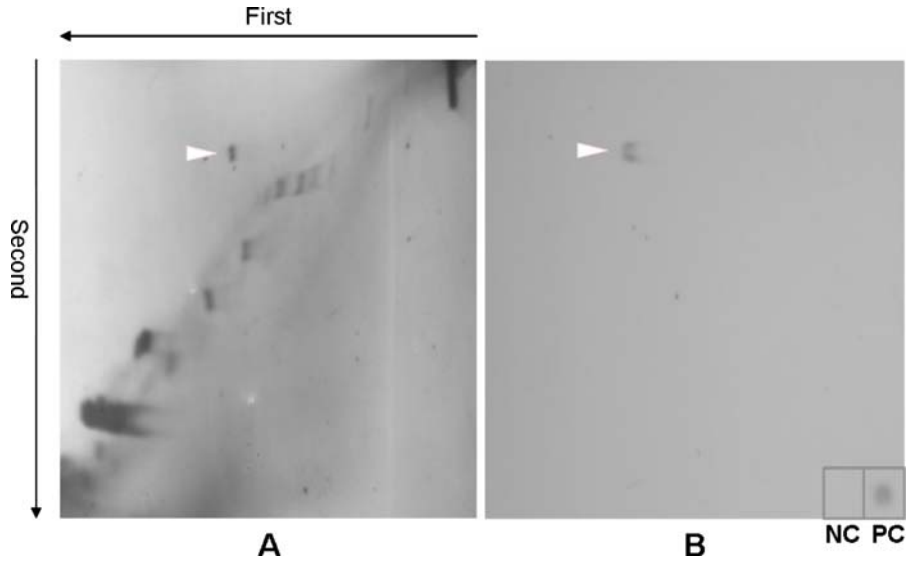

Fig. 1 2D-PAGE and Northern blot analysis of RNA extracts from jujube samples. **A** Total RNA was extracted from jujube samples collected from Taigu (TG1). Samples were separated by 2D-PAGE and the gel silver stained to visualise the bands present. **B** Hybridisation of (**A**) with a DIG-labelled RNA probe for HSVd. The white arrowhead indicates HSVd circular RNA. Horizontal and vertical arrows labelled as "First" or "Second" indicate the direction of electrophoresis applied during 2D-PAGE. PC: HSVd positive control (HSVd-infected cucumber); NC: Healthy cucumber

isolates were divided into three major groups (i.e. plum-type, hop-type, and citrus-type), and two minor groups created from recombination events that occurred between members of the three main groups (Kofalvi et al. 1997). Our phylogenetic analysis of the 49 total HSVd sequence variants showed that all 13 HSVd sequence variants obtained were included in the plum-type major group (Fig. 3).

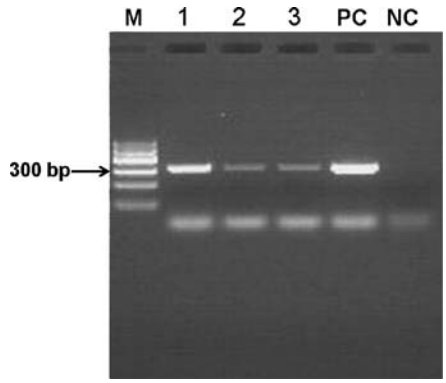

Fig. 2 Agarose gel electrophoresis of the HSVd RT-PCR products. HSVd amplified from TG1 (Lane 1), CZ3 (Lane 2), and CZ4 (Lane 3). Size markers are provided in Lane M with the 300 bp band indicated with an arrow. PC: HSVd positive control; NC: uninfected jujube control

In this study, we characterised HSVd isolates obtained from jujube trees in China. Sequencing results and phylogenetic analysis confirmed that the sequences obtained were highly conserved relative to other HSVd isolates (Amari et al. 2001). These results provide a foundation for further study of the origin, infectivity, and possible intermediate hosts of HSVd. Cross-infection due to farm operations such as pruning and grafting is very common in the transmission of viroids; therefore, it could be suspected that the HSVd isolated from jujube trees was the result of cross-infection. However, due to the absence of any other fruit tree near the orchard where the jujube samples were collected, the possibility of cross-infection is very unlikely. The jujube trees infected with HSVd did not show any obvious disease symptoms compared to the HSVd-free jujube trees, although it is possible that HSVd could present latent infections in jujube. Until further studies of climate, latitude, planting, and other variables are considered, as well as the source and pathogenicity of HSVd in jujube trees, no definitive conclusions can be made regarding the

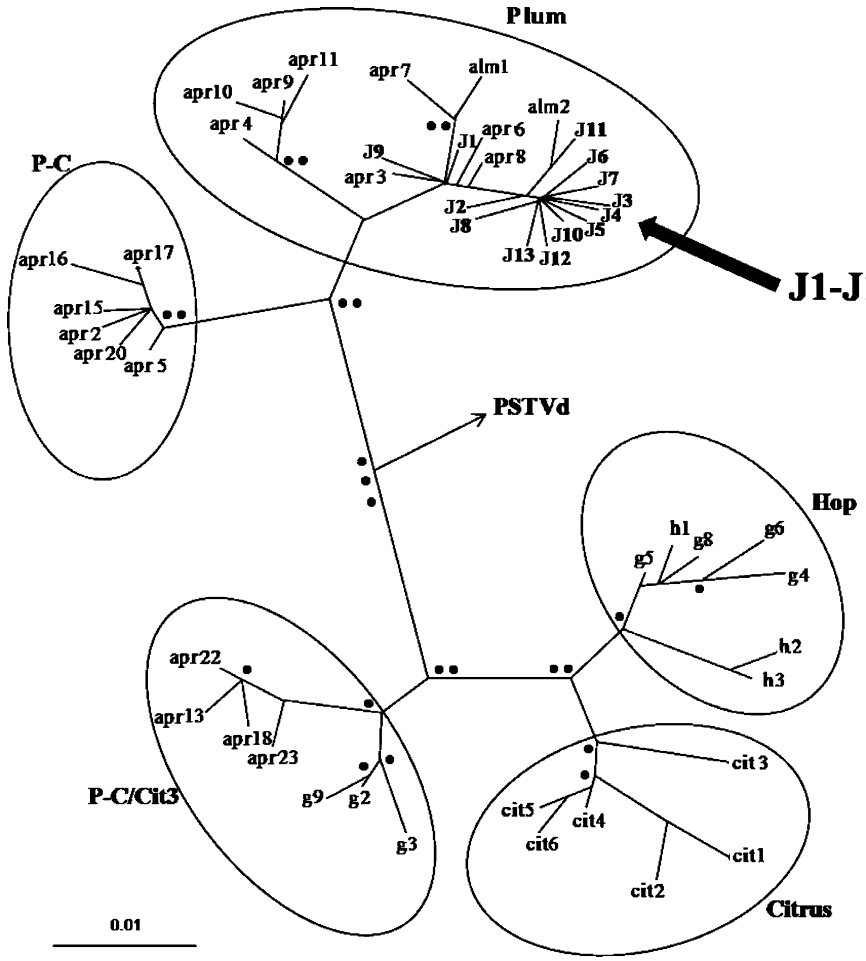

Fig. 3 Phylogenetic tree of HSVd sequence variants. HSVd variants isolated from jujube leaves (i.e. J1-J13) and representative HSVd sequences previously described were used to generate a phylogenetic tree based on genetic distances (PHYLIP 3.5c package) calculated among the HSVd sequence variants. Five phylogenetic groups were established as previously described (Amari et al. 2001). The HSVd sequence variants from jujube clustered into the Plum group. The position and branching of PSTVd, included in the analysis as an out-group, are indicated with an arrow. The statistical value of the nodes, as determined by bootstrap analysis using 100 replicates, are indicated by the dots associated with each node. Three dots (…) indicate detection in 100% of replicates, two dots (..) indicate detection in 80–100% of replicates, and one dot (.) indicates detection in 50% of replicates

ability of HSVd to cause disease symptoms in jujube trees. With a new host of HSVd established, the role of the jujube tree in further studies of HSVd, as well as other viroids, remains to be clarified.

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References

Amari, K., Gomez, G., Myrta, A., Di Terlizzi, B., & Pallás, V. (2001). The molecular characterization of 16 new sequence variants of Hop stunt viroid reveals the existence of invariable regions and a conserved hammerhead-like structure on the viroid molecule. *The Journal of General Virology, 82*, 953–962.

Astruc, N., Marcos, J. F., Macquaire, G., Candresse, T., & Pallás, V. (1996). Studies on the diagnosis of hop stunt viroid in fruit trees: identification of new hosts and application of a nucleic acid extraction procedure based on non-organic solvents. *European Journal of Plant Pathology, 102*, 837–846. doi:10.1007/BF01877053.

Canizares, M. C., Marcos, J. F., & Pallás, V. (1999). Molecular characterization of an almond isolate of hop stunt viroid (HSVd) and conditions for eliminating spurious hybridization in its diagnosis in almond samples. *European Journal of Plant Pathology, 10*, 553–558. doi:10.1023/A:1008794531725.

Diener, T. O. (1989). Subviral pathogens of plants: the viroids. *La Ricerca in Clinica e in Laboratorio, 19*, 105–128.

Diener, T. O. (2003). Discovering viroids - A personal perspective. *Nature Reviews Microbiology, 1*, 75–80. doi:10.1038/nrmicro736.

Flores, R., Di Serio, F., & Hernandez, C. (1997). Viroids: the noncoding genomes. *Seminars in Virology, 8*, 65–73. doi:10.1006/smvy.1997.0107.

Flores, R., Pallás, V., Khan, J., & Dijkstra, J. (2006). *Handbook of Plant Virology*. New York: The Haworth Press.

Flores, R., Randles, J. W., Bar-Joseph, M., & Diener, T. O. (1998). A proposed scheme for viroid classification and nomenclature. *Archives of Virology, 143*, 623–629. doi:10.1007/s007050050318.

Han, S. (2005). Specific primer for detection of Jujube Witches' Broom Phytoplasma group (16SrV) in Korea. *The Plant Pathology Journal, 21*, 55–58.

Hataya, T. (1999). Recent research in viroid diseases and diagnosis. *Recent Research Virology, 1*, 789–815.

Kofalvi, S. A., Marcos, J. F., Canizares, M. C., Pallás, V., & Candresse, T. (1997). Hop stunt viroid (HSVd) sequence variants from *Prunus* species: evidence for recombination between HSVd isolates. *The Journal of General Virology, 78*, 3177–3186.

Li, S. F., Onodera, S., Sano, T., Yoshida, K., Wang, G. P., & Shikata, E. (1995). Gene diagnosis of viroids: comparisons of return-PAGE and hybridization using DIG-labeled DNA and RNA probes for practical diagnosis of hop stunt, citrus exocortis and apple scar skin viroids in their natural host plants. *Annals of the Phytopathological Society of Japan, 61*, 381–390.

Pelchat, M., Rocheleau, L., Perreault, J., & Perreault, J. P. (2003). Subviral RNA: a database of the smallest known auto-replicable RNA species. *Nucleic Acids Research, 31*, 444–445. doi:10.1093/nar/gkg026.

Polivka, H., Staub, U., & Gross, H. J. (1996). Variation of viroid profiles in individual grapevine plants: novel grapevine yellow speckle viroid 1 mutants show alterations of hairpin I. *The Journal of General Virology, 77*, 155–161. doi:10.1099/0022-1317-77-1-155.

Sano, T., Hataya, T., Terai, Y., & Shikata, E. (1989). Hop stunt viroid strains from dapple fruit disease of plum and peach in Japan. *The Journal of General Virology, 70*, 1311–1319. doi:10.1099/0022-1317-70-6-1311.

Schumacher, J., Randles, J. W., & Riesner, D. (1983). A two-dimensional electrophoresis technique for the detection of circular viroids and virusoids. *Analytical Biochemistry, 135*, 288–295. doi:10.1016/0003-2697(83)90685-1.

Shikata, E. (1990). New viroids from Japan. *Seminars in Virology, 1*, 107–115.

Yang, Y. A., Wang, H. Q., Guo, R., Cheng, Z. M., Li, S. F., & Sano, T. (2006a). First report of Hop stunt viroid in apricot in China. *Plant Disease, 90*, 828. doi:10.1094/PD-90-0828C.

Yang, Y. A., Wang, H. Q., Guo, R., Cheng, Z. M., Sano, T., & Li, S. F. (2006b). First report of Hop stunt viroid in plum in China. *Plant Pathology, 56*, 339. doi:10.1111/j.1365-3059.2007.01524.x.

GenBank accession numbers

GenBank accession numbers for the sequence variants identified for HSVd isolated from Jujube are FJ771011-FJ771023.