SHORT COMMUNICATION

ARE THERE STRAINS OF *CHRYSANTHEMUM STUNT VIROID*?

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SUMMARY

Chrysanthemum stunt viroid (CSVd) has been detected or isolated from chrysanthemums or other host species in 26 countries. While strains (biological variants) have been described for many viroids, strains have not been described as such specifically for CSVd, despite the determination of 117 sequence variants of CSVd in 16 countries. Tomato has been described as an experimental host, either symptomless, or showing mild, or even severe symptoms, depending on the report; however, most of these studies were done with single isolates of CSVd and one cultivar of tomato plants. Thus, here we re-examined these issues by directly comparing the biological activity of three isolates of CSVd, one each from the USA, China and Australia, varying in their nucleotide sequence. We found that these isolates showed no differences in symptoms induced on either chrysanthemum or tomato plants, suggesting that much of the biological variation reported previously may have been due to cultivar differences and/or environmental effects.

Key words: chrysanthemums, viroids, viroid strains, Chrysanthemum stunt viroid, sequence variation.

Chrysanthemum stunt disease, first described in the USA (Dimock, 1947) in florist's chrysanthemum species (*Dendranthema* spp., formerly *Chrysanthemum* spp.), was later shown to be caused by a viroid (Diener and Lawson, 1973; Hollings and Stone, 1973), designated *Chrysanthemum stunt viroid* (CSVd). Since then, CSVd has been either detected or isolated in 26 countries, largely from *Dendranthema* spp., although natural infections by CSVd have also been described in *Ageratum* spp. (GenBank Accession No. Z68201), *Argyranthemum frutescens* (Menzel and Maiss, 2000; Marais *et al*., 2011; Torchetti *et al*., 2012), *Dahlia* spp. (Nakashima *et al*., 2007), *Pericallis hybrid* (GenBank Accession No. GQ174501), *Petunia hybrida* (Verhoeven *et al*., 1998), *Solanum jasminoides* (Verhoeven *et al*., 2006; GenBank Accession No. JF414238), and *Vinca major* (Nie *et al*., 2001). CSVd is related to a number of other viroids in the genus *Pospiviroid* of the family *Pospiviroidae*, which includes the type member, *Potato spindle tuber viroid* (PSTVd), as well as *Citrus exocortis viroid* (CEVd). CSVd shares 67-73% sequence identity with PSTVd and CEVd (Haseloff and Symons, 1981; Gross *et al*., 1882). While the partial or complete nucleotide sequences of 117 isolates and variant cDNA clones of two isolates of CSVd have been published and/or deposited with the GenBank, little is known about the relationship, if any, between sequence variation vs. pathogenicity and/or host range. In previous studies, differences in symptoms have been described either as occurring naturally on different cultivars of chrysanthemums, or after inoculation of one isolate to several cultivars (reviewed by Lawson, 1987; Bouwen and van Zaayen, 2003). For example, Chung *et al*. (2001) described differences in symptoms on chrysanthemum cv. Chunkwang by two Korean isolates differing in only two nucleotides; however, these plants were collected from different greenhouses containing natural infections and were not plants inoculated at the same time in the same laboratory. When such plants containing the two isolates were graft-inoculated to chrysanthemum cv. Mistletoe, they both induced the same symptoms on this cultivar (Chung *et al*., 2001). Similarly, differences in responses of tomato (*Solanum lycopersicum*) to infection by CSVd have been noted between laboratories working with different isolates, ranging from no infection (Hollings and Stone, 1973), or no symptoms (Niblett *et al*., 1978, 1980; Matsushita and Penmetcha, 2009), to mild symptoms (Chung *et al*., 2001) and even severe symptoms (Runia and Peters, 1980; Kryczyncki and Paduch-Cichal, 1987; Verhoeven *et al*., 1998). While one study examined the effects on CSVd sequence alterations after passage in different hosts (Matsushita and Penmetcha, 2009), in no studies to date have different, sequenced isolates of CSVd been compared by inoculation to a common chrysanthemum cultivar, to establish whether differences occurred in pathology and to determine if

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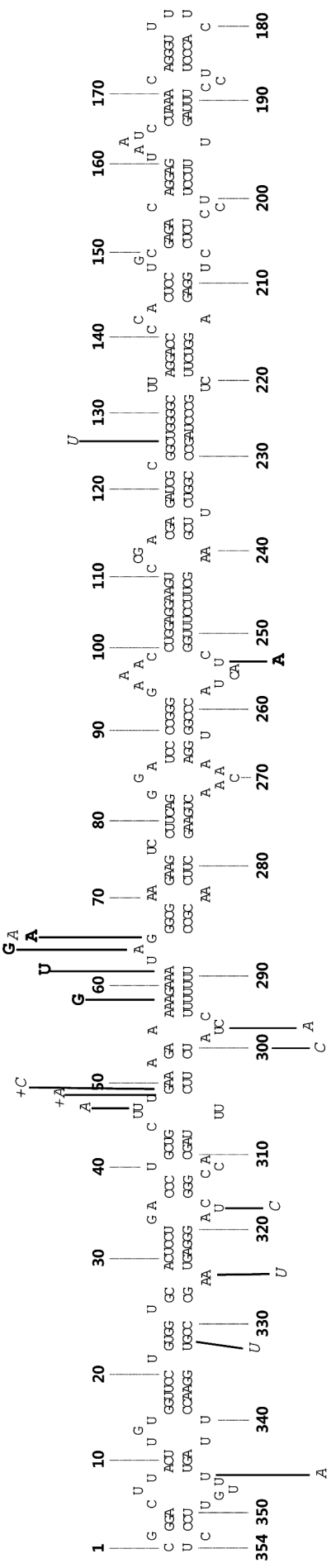

Fig. 1. Nucleotide sequence of CSVd isolate CN3. Sequence variation between CSVd-CN3 and CSVd-US1 is shown by bold letters. Sequence variation between CSVd-CN3 and CSVd-AU1 is shown by italic letters. A plus symbol before a letter indicates an insertion. CSVd-AU1 and CSVd-US1 differed from each other by substitutions at positions 47 (A>U), 58 (A>G), 62 (A>U), 126 (U>C), 254 (U>A), 300 (C>U), 318 (C>U), 326 (U>A), 332 (U>G), and 345 (A>U), respectively, as well by insertions in CSVd-AU1 between positions 48/49 (A) and 49/50 (C).

changes in nucleotide sequences could be related to such differences, as has been done with PSTVd in tomato (Keese and Symons, 1985; Schnölzer *et al*., 1985), *Coconut cadang-cadang viroid* in coconut (Rodriquez and Randles, 1993), CEVd (Murcia *et al*., 2011; Visvader and Symons, 1986) and *Hop stunt viroid* (Reanwarakom and Semancik, 1998, 1999) in citrus, *Chrysanthemum chlorotic mottle viroid* in chrysanthemum (De la Peña *et al*., 1999; De la Peña and Flores, 2002) and *Peach latent mosaic viroid* in peach (Malfitano *et al*., 2003). Thus, despite the wealth of nucleotide sequence data and biological analyses, there is little-to-no connection between the nature of sequence variation and pathogenicity for CSVd. Here, we describe an analysis of three CSVd isolates that were compared biologically, to evaluate whether they showed variation in their pathogenicities in two hosts, chrysanthemum and tomato.

The CSVd isolates tested were the Cornell University isolate of CSVd (CSVd-US1), described biologically in earlier studies (Dimock, 1947; Niblett *et al*., 1978, 1980), the sequence of which was determined by Müller-Derlich (1990) and is given in GenBank Accession number X16409; the Australia isolate of CSVd (CSVd-AU1), which was the first CSVd isolate sequenced (Haseloff and Symons, 1981) and is given in GenBank Accession No. V01107; and an isolate from Beijing, China, designated CSVd-CN3, for which we determined the nucleotide sequence (Fig. 1; GenBank Accession No. JQ809255). The three isolates differed from each other by 5 nt (CN3 vs. US1), 12 nt (AU1 vs. CN3), and 13 nt (AU1 vs. US1) (Table 1). The positions of the differences between CN3 vs. US1 and CN3 vs. AU1 are shown in Fig. 1, and the positions of differences between US1 vs. AU3 are given in the Fig. 1 legend. All but one of the changes between CN3 vs. AU1 or CN3 vs. US1 occurred in the left half of the molecule, as depicted in the rod-like conformation (Fig. 1). Also, all but one of the 5 nt differences between CN3 vs. US1 occurred in the upper strand between nucleotides 58 and 65, with one occurring in the lower strand (nucleotide 254). By contrast, half of the 12 nt differences between CN3 and AU1 occurred on the upper strand, with five occurring between nucleotides 47 and 65, and one at nucleotide 126, while the other 6 nt differences occurred in the lower strand between nucleotides 298 and 345 (Fig. 1). The CN3 isolate differed from the Type English CSVd isolate (Gross *et al*., 1982) at nucleotides 64, 65, 254 and 298, while the US1 isolate differed from the English CSVd isolate at nucleotides 58, 62 and 298. Thus, the Australian isolate contained a larger number of differences compared to the rest.

We compared these three isolates of CSVd for their pathogenic responses on chrysanthemum cv. Bonnie Jean. This chrysanthemum cultivar was chosen since it

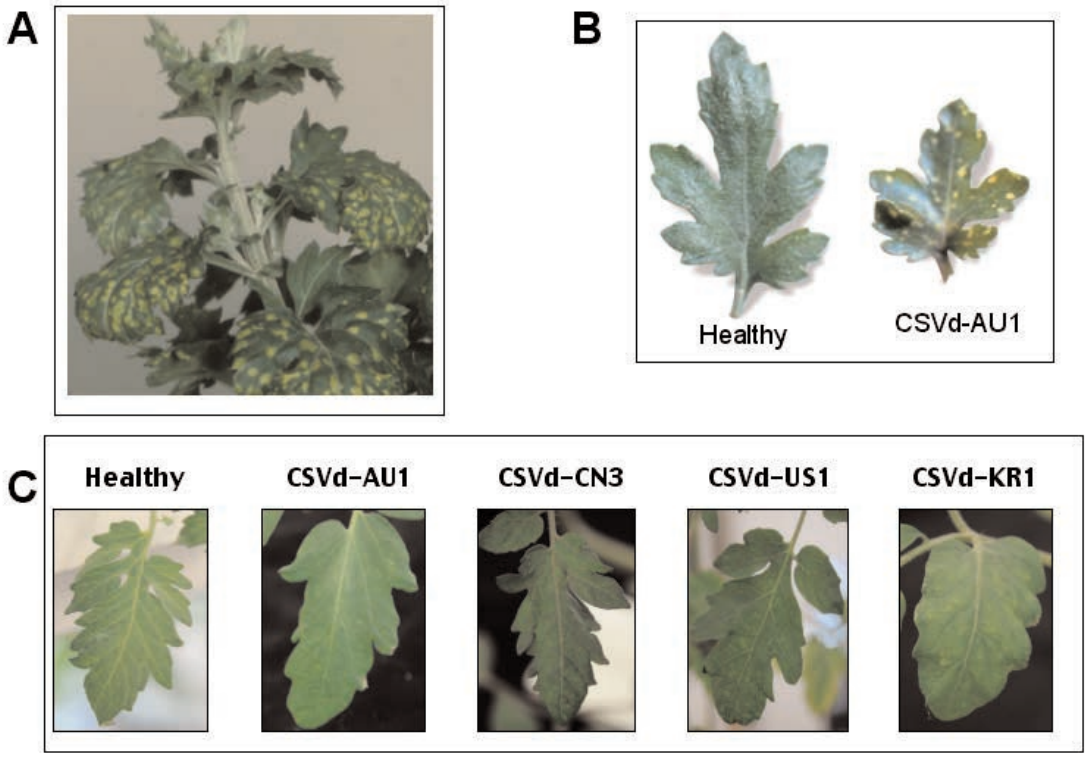

Fig. 2. Pathogenic responses of chrysanthemum and tomato plants to infection by CSVd isolates. Nucleic acids extracted from infected chrysanthemum plants infected by different isolates of CSVd (AU1 from Adelaide, Australia; CN3 from Beijing, China; US1 from Cornell University, USA; and KR1 from Masan, South Korea), were inoculated to (A) chrysanthemum cv. Bonnie Jean (shown for CSVd-US1), (B) chrysanthemum cv. Mistletoe (shown for CSVd-AU1), or (C) tomato plants of cv. Songsong-I (all four isolates), and were maintained for 4 weeks. Stunting and large chlorotic spots developed on chrysathemum cv. Bonnie Jean, while stunting and small, "measles-like" chlorotic spots developed on cv. Mistletoe. No stunting or leaf distortion was observed on the tomato plants, but a mild mottle developed on the tomato plants infected by all four isolates.

had previously been shown to exhibit differences in foliar symptoms after infection by CEVd, CSVd, *Chrysanthemum chlorotic mottle viroid* and two strains of PSTVd (Niblett *et al.*, 1978, 1980). The inocula consisted of nucleic acids extracts at 1 mg/ml, which contained similar levels of viroid, as determined by either gel electrophoresis or reverse-transcription polymerase chain reaction. The chrysanthemum plants inoculated with the three isolates by stem-slashing (Palukaitis and Symons, 1980) were maintained in soil with nutritional supplement in a growth chamber at 30ºC with a 16 h light/14 h dark period and were assessed for symptom responses at 4 weeks post-infection (wpi). All three isolates induced the same symptoms of stunting and large chlorotic lesions in cv. Bonnie Jean (shown for CSVd-US1 in Fig. 2A), the same as described previously for isolate CSVd-US1 (Niblett *et al.*, 1980). After inoculation to chrysanthemum cv. Mistletoe and incubation at 24-25ºC with a 16/8-h light-dark period, all three isolates developed stunting and the "measles" type of chlorotic lesions (not shown), also seen on cv. Mistletoe when incubated at 28ºC with a 14/8-h dark-light period (shown for isolate CSVd-AU1 in Fig. 2B), and as described for other isolates by other researchers using this cultivar (Dimock, 1947; Hollings and Stone, 1973; Lawson, 1987; Chung *et al.*, 2001). Thus, among these three isolates, there were no differences in pathogenicity on the tested chrysanthemum cultivars.

The same three isolates (CSVd-AU1, -CN3 and -US1), also were inoculated to tomato cv. Rutgers plants by rubbing the nucleic acid extracts onto the cotyledons. The inoculated plants were maintained in a growth chamber at 30ºC with a 16/8-h light-dark period and were examined for symptoms and viroid accumulation at 2 to 4 wpi. The three isolates analyzed here all replicated to similar levels without inducing symptoms in tomato cv. Rutgers (data not shown), as reported pre-

Table 1. Variation in sequence between CSVd isolates AU1, CN3, and US1.

Number of nucleotide differences between isolates			
Isolate Name	AU1	CN3	US1
AU1	—	12	13
CN3	12	—	5
US1	13	5	—

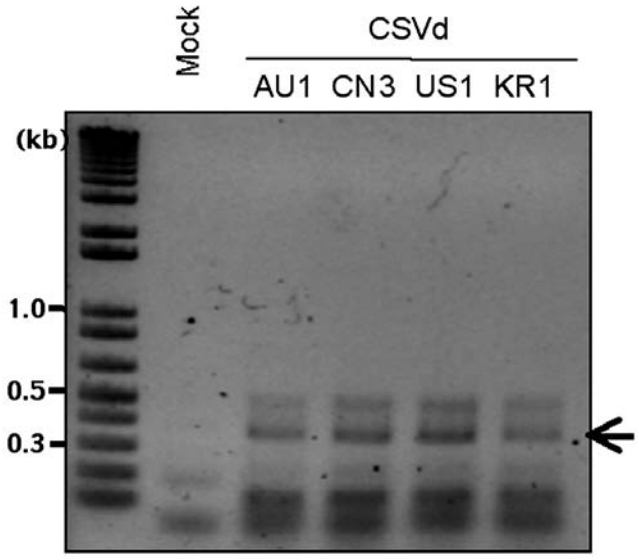

Fig. 3. RT-PCR analysis to confirm the infectivity of four CSVd isolates on tomato plants. Total RNAs were extracted from tomato cv. Songsong-i inoculated with CSVd isolates AU1, CN3, US1, and KR1, as well as from mock-inoculated plants. The RNAs were subjected to RT-PCR and the PCR products were analyzed by electrophoresis in a 1% agarose gel, along with a DNA marker ladder, and stained with ethidium bromide. The position of the expected size PCR product is indicated by an arrow.

viously for isolate CSVd-US1 (Niblett *et al*., 1978, 1980). In separate experiments, the three CSVd isolates (CSVd-AU1, -CN3, -US1) were inoculated to tomato plants of cv. Songsong-i along with the Korean isolate CSVd-KR1 and were maintained in a growth room at a lower temperature: 20-23ºC with a 16/8-h light-dark period. Four wpi, all plants showed the same symptom response: a mild mottle with no stunting or leaf distortion (Fig. 2C). The four viroid isolates also all accumulated to similar levels (Fig. 3). Thus, for this group of CSVd isolates, from four different countries, differences in symptoms obtained appeared to be related more to cultivar differences and/or growth conditions than to specific sequence differences among isolates.

Two Korean isolates both were reported to induce mild mosaic symptoms in tomato cv. Rutgers (Chung *et al*., 2001), while a Japanese isolate, which had the same sequence as one of the Korean isolate (CSVd-KR1), did not induce symptoms in tomato plants for which the cultivar was not specified, when maintained at 20-24ºC under a long photoperiod (Matsushita and Penmetcha, 2009). This Japanese CSVd isolate, which also had the same nucleotide sequence as the original English CSVd isolate (Gross *et al*., 1982), showed eight nucleotide sequence changes scattered around the genome after propagation of a cDNA clone in tomato plants (Matsushita and Penmetcha, 2009). Thus, while sequence changes may be induced after passage in tomato plants, there is also no clear relationship between specific sequence differences in CSVd isolates and changes in pathogenicity in tomato.

Overall, our data indicate that the changes in nucleotide sequence observed between four isolates of CSVd, obtained from four different countries, did not result in differences in symptoms on two cultivars of either chrysanthemum or tomato. This is on contrast to isolates of PSTVd (Niblett *et al*., 1978, 1980). Whether other cultivars of chrysanthemum or tomato could be used to differentiate biological variants of CSVd under some yet-to-be-defined environmental condition is not known. Thus, the question of whether there are biological variants of CSVd still remains open.

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