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), Argyre- 
thaenium frutescens (Menzel and Maiss, 2000; Marais et al., 2011; Torchetti et al., 2012), Dalia 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 Pospi- viroid of the family Pospi-viridaceae, 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; Kryczynski 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
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 (Maltitano 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
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 (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 chrysanthemum 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.

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 prev-

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<th>Isolate Name</th>
<th>AU1</th>
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<tr>
<td>AU1</td>
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<td>CN3</td>
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<td>US1</td>
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Are there strains of CSVd?

Previously 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: 20-23°C 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 20-24°C under a long photoperiod (Matsushita and Pennetche, 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 Pennetche, 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 in 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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