Nucleotide Sequences and Distribution of Chrysanthemum Stunt Viroid in Japan

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We assayed for Chrysanthemum stunt viroid (CSVd) in cultivated chrysanthemum (Chrysanthemum morifolium) collected from 10 prefectures in Japan and 8 wild species (Chrysanthemum spp.) cultivated at the National Institute of Floricultural Science (NIFS), Japan and determined complete nucleotide sequences of CSVd isolates infecting plants. CSVd was detected in 80 of 89 samples of cultivated chrysanthemum, and samples from all prefectures were infected. Since all 8 wild species had CSVd in RT-PCR results, they were recognized as hosts of CSVd, even though no stunt symptoms were observed. Five sequence variants of CSVd were distinguished among the 21 isolates based on the difference of the nucleotide sequences. Mutations were common in the P (pathogenicity) domain. Variant 5 from C. morifolium, and variant 4 from C. yoshinaganthum had different nucleotide sequences from those reported previously. Variant 1 was most frequently detected from samples in 6 prefectures and is assumed to be the predominant CSVd variant distributed in Japan.

Key Words: Chrysanthemum stunt viroid (CSVd), distribution, sequence variants.

Introduction

Stunting caused by Chrysanthemum stunt viroid (CSVd) is one of the most damaging diseases of cultivated chrysanthemum (Chrysanthemum morifolium Ramat. = Dendranthema grandiflorum Kitam.), the most important cut flower in Japan. This disease has been reported in many areas in the world (Bouwen and Zaayen, 2004). The symptoms are severe, with stunting of plant height, reduced flower size, and flower color bleaching (Horst et al., 1977). The causal viroid, CSVd, is a small, single-stranded, infectious RNA of 348–356 nucleotides (nt) forming a circular secondary structure, and belonging to the family Pospiviroidae with Citrus exocortis viroid and Potato spindle tuber viroid. The replication and circularization of the viroid are dependent on the host, and the viroid is transmitted in plant sap or through grafting. It can also infect Senecio cineraria (Lawson, 1968), also in the Compositae, and Petunia hybrida (Solanaceae) is a natural host (Verhoeven et al., 1998).

Since the first detection of CSVd in Japan (Osawa et al., 1977), it has been reported in cultivated chrysanthemums in the prefectures of Mie (Hanada et al., 1982), Kagawa (Kusunoki et al., 1993), Hyogo (Shiwaku et al., 1996), Kumamoto (Moriyama et al., 1996), Hokkaido (Li et al., 1997), Yamagata (Kanematsu, 1998), Niigata (Sugiura and Hanada, 1998), and Shizuoka (Doi and Kato, 2004); however, its distribution and the range of variation in the complete nucleotide sequence have not yet been clearly shown in Japan.

The objective of this study was to assay for CSVd in chrysanthemum plants collected in various areas in Japan and to completely sequence the viroid to show the distribution of CSVd sequence variants in Japan.

Materials and Methods

Collection of chrysanthemum plants

During 2005–2006, we collected 89 samples of large-flowered, spray-type, and small-flowered cultivated chrysanthemums with symptoms of stunt or discoloration, or suggestive of infection from 10 prefectures in Japan (Table 1). The cultivars were representative of those grown in each prefecture. We also collected 10 samples in 2005 from 8 wild species maintained at the National Institute of Floricultural Science, Tsukuba, Japan (NIFS): C. crassum (Kitam.) Kitam., C. indicum L. var. indicum, C. japonense Nakai var. japonense, C. makinoi Matsum. & Nakai, C. wakasaense Shimot. ex Kitam., C. weyrichii (Maxim.) Miyabe & T. Miyake, C. yoshinaganthum Makino ex Kitam., and C. zawadskii Herbich.
CSVd detection and nucleotide sequence determination

RT-PCR and nested PCR were carried out according to Hosokawa et al. (2005) with some modifications, with the primers CSVd-R and CSVd-F, and CSVd-NR and CSVd-NF, respectively (Table 2). RNA samples were collected with a syringe needle (25G × 25 mm, Terumo, Tokyo, Japan) from the basal part of leaves, previously described as “the direct microtissue sampling method” (Hosokawa et al., 2005). Adherent templates were mixed with an RT (reverse transcription) reaction mixture by dipping the needle for 30 s. The RT mixture contained 2 µL of RT buffer, 1 µL of dNTPs (10 mM), 0.5 µL of reverse primer (20 µM), 0.5 µL of RNase inhibitor (1 U, Toyobo, Osaka, Japan), 0.5 µL of reverse transcriptase (20 µM), and distilled water to a final volume of 9 µL. The RT mixture was incubated at 42 °C for 30 min and then at 99 °C for 5 min, and used as a template for PCR. The PCR mixture was composed of 1.6 µL of RT product, 0.1 µL of forward primer solution (20 µM), 1 µL of reaction buffer, 0.1 µL of KOD Dash polymerase (Toyobo), 1 µL of dNTPs (2 mM), and distilled water to a final volume of 10 µL. The PCR conditions were 35 cycles of 45 s of melting at 98 °C, 10 s of annealing at 62 °C, and 45 s of extension at 74 °C. The RT-PCR product (6 µL) was separated by electrophoresis in 1% agarose gel, stained with ethidium bromide, and visualized under UV light. PCR products that did not match the expected bands corresponding to the viroid were used as nested PCR templates. The nested PCR mixture contained 1 µL of RT-PCR product, 1 µL of dNTPs (2 mM), 0.1 µL each of forward and reverse primer solution (20 µM), 1 µL of KOD Dash polymerase, and distilled water to a final volume of 10 µL. The nested PCR conditions were 30 cycles of 30 s at 94 °C, 5 s at 60 °C, and 30 s at 74 °C. Nested PCR product (6 µL) was analyzed by means of 1% agarose gel electrophoresis. RT and PCR were conducted in a PCR device (Dice, Takara, Otsu, Japan). The detection of a band of about 250 bp by RT-PCR indicated CSVd infection at a high concentration, and that of about 200 bp by nested PCR revealed the presence of CSVd at a low concentration.

For nucleotide sequencing of 14 samples of cultivated chrysanthemum and 7 of wild chrysanthemum infected at a high concentration, we used CSVd-R and CSVd-F primers according to the method of Hosokawa et al. (2005), and CSV-1M and CSV-1P according to the method of Li et al. (1997) (Table 2). RT-PCR was conducted using the RT templates from infected plants detected by RT-PCR, as described above. After gel electrophoresis of RT-PCR products, amplified cDNA fragments were purified with a Qiagen QIA Quick gel extraction kit and directly sequenced with a DNA

Table 1. PCR detection of CSVd in cultivated chrysanthemums collected from various areas in Japan during 2005–2006.

| Collected area | Number of samples | Number of CSVd-infected samples with high concentration (detected by RT-PCR) | Number of low concentration (detected by nested PCR) | Number of CSVd-free samples (not detected by nested PCR) |
|---------------|-------------------|-------------------------------------------------|-----------------------------------------------------|------------------------------------------------------|
| Fukushima     | 15                | 3                                               | 12                                                  | 0                                                    |
| Niigata       | 5                 | 2                                               | 3                                                   | 0                                                    |
| Ibaraki       | 40                | 10                                              | 9                                                   | 9                                                    |
| Tochigi       | 3                 | 3                                               | 9                                                   | 0                                                    |
| Gunma         | 12                | 9                                               | 0                                                   | 0                                                    |
| Kanagawa      | 2                 | 0                                               | 2                                                   | 0                                                    |
| Mie           | 4                 | 2                                               | 0                                                   | 0                                                    |
| Okayama       | 3                 | 3                                               | 0                                                   | 0                                                    |
| Kagawa        | 1                 | 1                                               | 0                                                   | 0                                                    |
| Fukuoka       | 4                 | 3                                               | 1                                                   | 0                                                    |
| Total         | 89                | 36                                              | 44                                                  | 9                                                    |

Table 2. Oligonucleotide primers used for the detection and sequence determination of CSVd.

| Name  | Method      | Sequence (5'–3') | Target of amplification (position number) | PCR product size | Reference       |
|-------|-------------|------------------|------------------------------------------|------------------|-----------------|
| CSVd-R| RT-PCR      | AGGATTACTCTCTGTCTCGCA | 148–167                                  | 253              | Hosokawa et al. (2005) |
| CSVd-F| RT-PCR      | CAACTGAAAGCTCAAGCCTT | 269–290                                  | 204              |                 |
| CSVd-NR| Nested PCR | AGTGGGGTCTAAAGCCCAA | 126–145                                  |                  |                 |
| CSVd-NF| Nested PCR | CCAATCTTCTTTTAGCACC | 296–315                                  |                  |                 |
| CSV-1P| RT-PCR      | CTTAGGACCCACTCTCG | 132–151                                  | 349              | Li et al. (1997) |
| CSV-1M| RT-PCR      | CGCGATCTCGTGAGACTTC | 125–106                                  |                  |                 |
| CSVd-ZR| Sequence   | GGAACCACAAGTAACTCCCG | 1–20                                     | 354              | This study      |
| CSVd-ZF| Sequence   | TGTGTTGCACTCTGACCT | 21–40                                    |                  |                 |
sequence (isolated from Chenopodium rubrum) with a length of 354 nt, registered as CSVd-142 (Table 1). The 354 nt sequences were subjected to RT-PCR using primer sets CSVd-ZF and CSVd-ZR developed in this study. The PCR product was analyzed on a 2% agarose gel and purified with QIAQuick gel extraction kit (Qiagen, Valencia, CA). The purified DNA was cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and five clones of each PCR product were sequenced. Some of the nucleotide sequences were submitted to the DDBJ database, as shown in Table 3. The nucleotide sequence length of CSVd obtained from 14 infected cultivated chrysanthemum and 7 wild chrysanthemum samples was 354 nt, the same as reported from Kumamoto Prefecture (Kanematsu, 1998), and Shizuoka Prefecture (Doi and Kato, 2004). Thus, CSVd should infect wild species of chrysanthemum without symptoms.

**Complete sequences of CSVd in infected chrysanthemum plants in Japan**

The nucleotide sequence length of CSVd obtained from 14 infected cultivated chrysanthemum and 7 wild chrysanthemum samples was 354 nt, the same as reported from Hyogo Prefecture (Shiwaku et al., 1996), Hokkaido Prefecture (Li et al., 1997), Niigata Prefecture (Sugiura and Hanada, 1998), Yamagata Prefecture (Kanematsu et al., 1998), and Shizuoka Prefecture (Doi and Kato, 2004), but shorter than the 356 nt reported from Kumamoto Prefecture (Kanematsu, 1998). The reason why only samples collected in Ibaraki Prefecture were free of CSVd is unknown, but this could be explained by the fact that samples with less-certain signs of infection were more common in this prefecture. CSVd was also detected in all 8 wild species tested by RT-PCR (data not shown), which have not previously been reported as hosts of CSVd. Yet even at high concentrations, no stunt symptoms were apparent. CSVd-infected plants of tomato (Lycopersicon esculentum Mill.) and petunia (Petunia hybrida Vilm.) also show no symptoms (Niblett et al., 1980; Verhoeven et al., 1998). Thus, CSVd should infect wild species of chrysanthemum without symptoms.

**Results and Discussion**

**CSVd detection in chrysanthemum plants collected from various prefectures in Japan**

RT-PCR detected CSVd in 36 of 89 samples of cultivated chrysanthemum and nested PCR detected CSVd in 44 of the remaining 53 samples (Table 1). CSVd was not detected in only 9 samples, all from Ibaraki Prefecture, including some suspicious samples. Since no prefectures were free of CSVd, the pathogen is probably present in all chrysanthemum, growing regions in Japan. This is the first report of CSVd with complete sequences from Fukushima Prefecture, Ibaraki Prefecture, Tochigi Prefecture, Gunma Prefecture, and Fukuoka Prefecture. It supports the results of our survey of plant protection officers on disease occurrence in each prefecture (Matsushita, 2006). The pathogen was detected in all flower types suggesting that all cultivated chrysanthemums are susceptible to the disease. The reason why only samples collected in Ibaraki Prefecture were free of CSVd is unknown, but this could be explained by the fact that samples with less-certain signs of infection were more common in this prefecture. CSVd was also detected in all 8 wild species tested by RT-PCR (data not shown), which have not previously been reported as hosts of CSVd. Yet even at high concentrations, no stunt symptoms were apparent. CSVd-infected plants of tomato (Lycopersicon esculentum Mill.) and petunia (Petunia hybrida Vilm.) also show no symptoms (Niblett et al., 1980; Verhoeven et al., 1998). Thus, CSVd should infect wild species of chrysanthemum without symptoms.

**Table 3. Comparison of nucleotide sequences of CSVd isolates from stunted chrysanthemum in Japan.**

| Variant number | Isolated area (Prefecture) | Number of isolates | Hosts                                      | Position of mutation | Accession number in DDBJ (Reference) |
|---------------|---------------------------|-------------------|-------------------------------------------|----------------------|-------------------------------------|
| 1             | Tsukuba (Ibaraki), Ishioka (Ibaraki), Kashima (Ibaraki), (Fukushima), (Tochigi), Agatsuma (Gunma), (Okayama), (Fukuoka) | 14                | *Chrysanthemum morifolium*, *C. japonense var. japonense*, *C. weyrichii* | U G A G A G G C U A A U | X16408 (Shiwaku et al., 1996) |
| 2             | Tsukuba (Ibaraki)          | 4                 | *C. japonense var. japonense*, *C. indicum var. indicum*, *C. makinoi*, *C. zawadskii* | U G A G A G G C U A A U | D88895 (Kanematsu et al., 1998) |
| 3             | Ishioka (Ibaraki)          | 1                 | *C. morifolium*                          | A A C G A G C U U A A U | M19506 |
| 4             | Tsukuba (Ibaraki)          | 1                 | *C. yoshinagananthum*                    | U G A A G G C U A A U | AB279770 (Li et al., 1997) |
| 5             | Tsukuba (Ibaraki)          | 1                 | *C. morifolium*                          | U A C G A G C U U A U | AB279771 (Li et al., 1997) |
| 6             | (Hokkaido)                 | 1                 | *C. morifolium*                          | U A U G A G U U U U A | AB006737 (Li et al., 1997) |
| 7             | (Shizuoka)                 | 1                 | *C. morifolium*                          | U G A G A G C C U U A | (Doi and Kato, 2004) |
| 8             | (Niigata)                  | 1                 | *C. morifolium*                          | U G A G A C C U U U A | (Sugiura and Hanada, 1998) |

* Data from previous reports.

Position number is based on the sequence of strain 1. P domain is located in shadowed areas.

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Haseloff, J., and Symons, T. (1981). J. Gen. Virol. 60, 335–337. 2007.
We distinguished 5 sequence variants of CSVd among the isolates (Table 3). Variant 5, from *C. morifolium* (AB279771), and variant 4, from *C. yoshinaganthum* (AB279770), differ from those previously reported. Since all variants were otherwise identical in the results of the cloning analyses of the whole sequences, this confirms the stability of the sequences of these variants. Fourteen of the 21 isolates were variant 1, and their nucleotide sequence completely coincided with that of the isolate previously reported from Hyogo (Shiwaku et al., 1996). Since variant 1 was the most frequently isolated and was obtained from 6 prefectures, we assume this variant to be predominant in Japan. The sequences of variants 2 and 3, both from Ibaraki, completely coincided with, respectively, accessions M19506, from *Senecio cineraria* (Gross et al., 1982), and D88895, from chrysanthemum in Yamagata (Kanematsu et al., 1998). Variants 4 and 5, new variants, were isolated from Ibaraki, but at low frequency. CSVd was detected for the first time in all 8 species of wild chrysanthemum tested such as variant 1 from *C. japonense* var. *japonense* and *C. weyrichii*, variant 2 from *C. japonense* var. *japonense*, *C. indicum* var. *indicum*, *C. makinoi* and *C. zawadskii*, and variant 4 from *C. yoshinaganthum*. It is unknown whether CSVd infected wild chrysanthemums during cultivation at NIFS or was derived from wild stocks.

**Poospiviroidae**, including CSVd, have a rod-like structure which is divided into 5 structural or functional domains: central (C), pathogenic (P), variable (V), terminal right (TR), and terminal left (TL) (Keese and Symons, 1985). C, TR, and TL are highly conserved. Sequence analysis showed no mutations in these domains in any of the 21 isolates in this study. Some mutations were found in the P domain, located at nt 45–70 and 284–309 (Keese and Symons, 1985), as shown in Table 3. P domains in *Potato spindle tuber viroid* and closely related viroids are reported to be related to pathogenicity (Schnolzer et al., 1985). Other determinants located in different domains are assumed to concurrently regulate pathogenicity, as seen in *Tomato apical stunt viroid* and *Citrus exocortis viroid* (Sano et al., 1992). The relationship of the P domain with the pathogenicity of CSVd is not clear, because the pathogenicity of the variants differing in P domain sequences has not been investigated. Further studies are necessary to elucidate the relation between sequence variants and pathogenicity of CSVd to chrysanthemums.

The variants and distribution of CSVd from chrysanthemums in Japan were clarified for the first time in this study. It is interesting that variant 1 is predominant in various prefectures, and variants 4 and 5 are newly reported in Japan. To further clarify the distribution and composition of variants, further studies will be carried out in the near future.

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