Plant Pathol. J. 17(4) : 194-200 (2001)

The Plant Pathology Journal

Chrysanthemum stunt viroid in *Dendranthema grandiflorum*

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(Received on June 1, 2001)

Chrysanthemum stunt viroid **(CSVd) was identified in chrysanthemum cv. Chunkwang showing symptoms of stunt with leaf distortion (K1) and stunt with chlorosis of leaves (K2) collected from the main cultivation area of Masan, Kyongnam province in Korea. The specific RNAs related with the diseased chrysanthemums were detected. Full-length 354 bp CSVd cDNAs were amplified from infected tissue by reverse transcription and polymerase chain reaction using a pair of primers specific for CSVd sequence. The amplified cDNA products were analyzed by agarose gel electrophoresis and the specific cDNAs were cloned. Nucleotide sequences of the two CSVd isolates K1 and K2 varied. Phylogenetic analysis of the nucleotide sequences of CSVd isolates indicated that K1 was closely related with J2 and Am2 isolates. K1 and K2 were transmitted by grafting to *Dendranthema grandiflorum* cv. Mistletoe, *Gynura aurantiaca*, and *Lycopersicon esculentum* cv. Rutgers. This is the first report of CSVd in *D. grandiflorum* in Korea.**

Keywords **:** chrysanthemum, *Chrysanthemum stunt viroid*, RT-PCR.

Chrysanthemum is grown all over Korea for cut flower. Propagation is usually done by stem cutting, and infected plants are continuously reused. From pinch or cuttings, pathogens can be transmitted easily. *Chrysanthemum stunt viroid* (CSVd) (Mayo, 1999) was first observed in 1945, and then recognized as an infectious disease agent based on graft transmission (Olson, 1949). Nowadays, the pathogen occurs prevalently worldwide (Lawson, 1987). CSVd causes serious losses in 3 or 4 years after its first infection. A typical reaction in chrysanthemum cultivars is stunt with a reduction of one-half to two-thirds relative to the normal plant height (Bouwen and Annemarie, 1995; Diener and Lawson, 1973; Hooftman et al., 1996; Horst and Nelson, 1996).

CSVd is an infectious single-stranded circular RNA. CSVd RNA nucleotides of the American (Genbank accession AJ001850), Japanese (Shiwaku, 1996a), and Australian isolates (Genbank accession M19505, J02067; Haseloff and Symons, 1981) were 355, 354 and 356, respectively. Large-scale purification of CSVd was developed for the direct detection of CSVd by polyacrylamide gel electrophoresis of nucleic acid extracted from diseased tissue (Palukaitis and Symons, 1980). Reverse transcription and polymerase chain reaction (RT-PCR) assays were developed for the detection and identification of CSVd from sap or total nucleic acid extracts of infected chrysanthemum leaves (Hooftman et al., 1996; Shamloul et al., 1995a; Shamloul et al., 1995b; Shiwaku et al., 1996a).

In this study, CSVd was identified in field-grown chrysanthemums based on symptomatology, bioassay, RT-PCR, and nucleotide sequence determination.

Materials and Methods

CSVd source. Chrysanthemum cv. Chunkwang plants showing different disease symptoms of stunt with leaf distortion (K1) or stunt with chlorosis of leaves and mottle on leaves (K2) were collected from greenhouses in Masan, Kyongnam province, Korea (Figs. 1 and 2). CSVd RNA of Japanese isolate (J2) provided by Kuniko Shiwaku (Hyogo Prefectural Agricultural Institute, Befu, Kasai, Japan) was used as a standard viroid.

Infectivity bioassay. Indicator plants used were *Gynura aurantiaca*, *Lycopersicon esculentum* cv. Rutgers, and chrysanthemum cv. Mistletoe grown in a glasshouse at 28-30°C. *G. aurantiaca* and *L. esculentum* were inoculated mechanically with crude sap made by grinding leaf tissues from naturally K1 and K2 CSVd infected chrysanthemum in 0.01 M sodium phosphate buffer, pH 7.2, with a mortar and pestle. Chrysanthemum cv. Mistletoe known as a susceptible indicator plant for CSVd was grafted on naturally CSVd K1 and K2 infected chrysanthemum cv. Chunkwang plants (Olson, 1949). Reactions of the treated plants were investigated 2 months after mechanical inoculation or graft.

Extraction of total nucleic acids. Total nucleic acids were extracted from 200 g of leaves of healthy, K1 or K2 infected chrysanthemums 3 months after mechanical inoculation. To obtain CSVd K1 or K2 infected chrysanthemums, *L. esculentum* was inoculated with crude sap from the leaves of naturally CSVd K1 or K2 infected chrysanthemum cv. Chunkwang. Chrysanthemum seedlings were again inoculated with crude sap from the leaves of K1 or K2 CSVd infected *L. esculentum* (Palukaitis and Symons,

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1980). Extracted total nucleic acids were suspended in 3 ml of distilled water. Thirty (30) microliters of the total nucleic acids were fractionated on polyacrylamide gel. Electrophoresis was done with 5% acrylamide and 0.25% methylene bisacrylamide in a buffer of 40 mM-tris-acetate, pH 8.1, 20 mM sodium acetate, and 2 mM EDTA at the electric field of 10 mA/gel for 13 h (Loening, 1967). Nucleic acids were stained with ethidium bromide or with silver. Total nucleic acids from 200 g of infected chrysanthemum plants were diluted 1 : 1,000 (wt/vol) with distilled water and were quantitated spectrophotometrically by scanning from 220 nm to 300 nm.

CSVd RNA extraction. Total RNAs were extracted from the leaf tissues (1-2 g/sample) of healthy and naturally CSVd infected Chunkwang plants showing stunt with leaf distortion symptom or

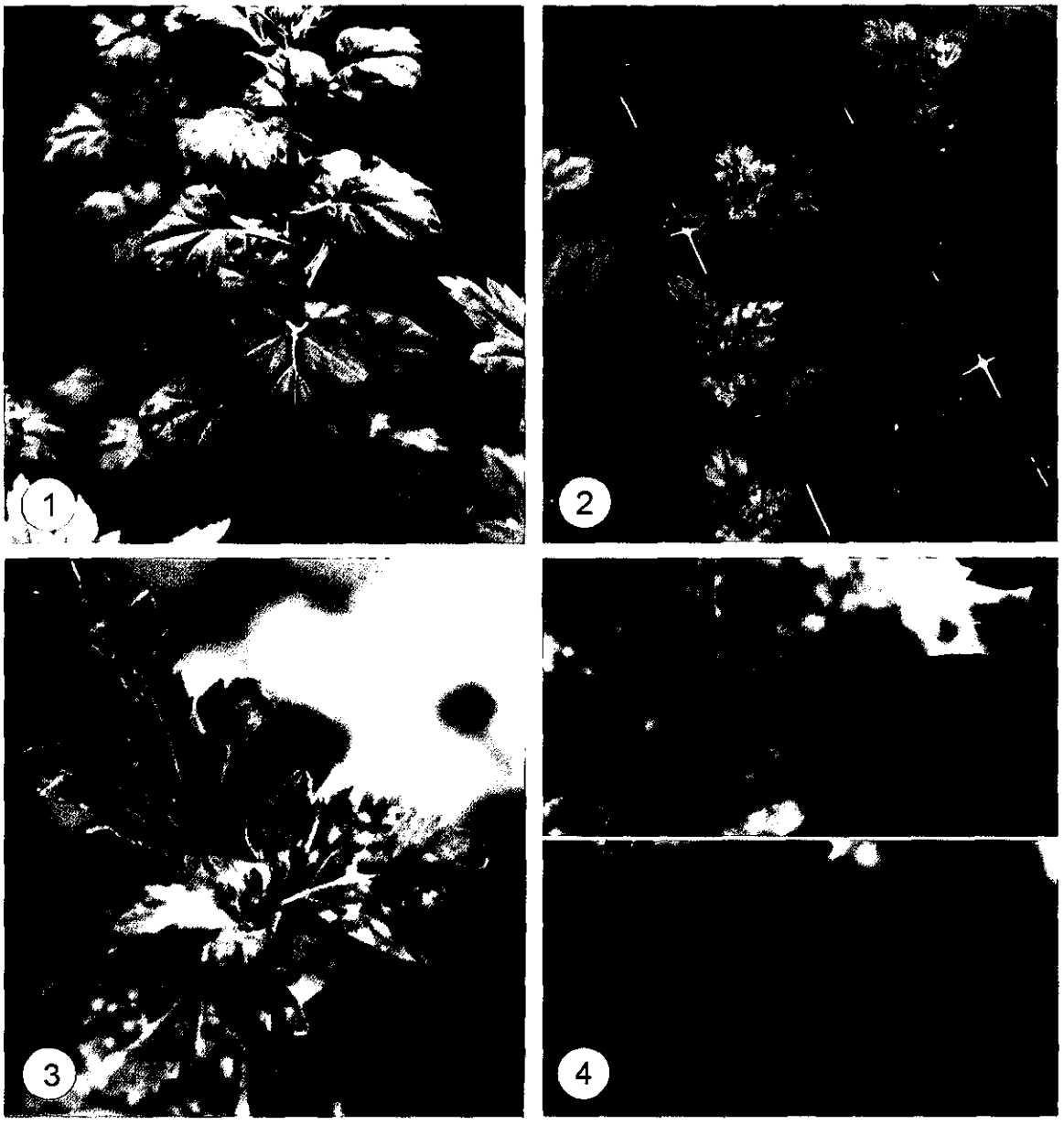


Fig. 1. *Chrysanthemum stunt viroid*-infected chrysanthemum cv. Chunkwang plants showing stunt with leaf distortion (K1).

Fig. 2. *Chrysanthemum stunt viroid*-infected chrysanthemum cv. Chunkwang plants showing chlorosis of leaves and mottle on leaves (K2).

Fig. 3. Chrysanthemum cv. Mistletoe showing yellow spots on leaves two months after graft on naturally CSVd K1-infected and K2-infected chrysanthemum cv. Chunkwang.

Fig. 4. *Lycopersicon esculentum* 'Rutgers' showing mosaic symptoms (upper part) and leaf distortion (lower part) two months after inoculation with crude sap of K1-infected or K2-infected chrysanthemum cv . Chunkwang.

stunt with chlorosis of leaves and mottle on leaves collected from different greenhouses in Masan for RT-PCR. Leaf samples were ground by using sterile mortars and were suspended in solution mixture containing 1.2 ml of extraction buffer (100 mM Tris-HCl, 50 mM EDTA, 2.8 M NaCl, pH 8.0), 60 µl of 20% SDS, 0.05 g polyvinylpyrrolidone, 1 µl of mercaptoethanol, and 600 µl of phenol : chloroform:isoamyl alcohol = 25 : 24 : 1 using the procedure of Shiwaku (1996b).

RT-PCR. A pair of CSVd specific primer was designed to amplify a 354 bp DNA fragment based on the nucleotide sequence of CSVd isolate (Am2; Genbank accession no. AJ001850). Antisense primer (5'-CCC TGA AGG ACT TCT TCG CC-3') was complementary to nucleotides 68-87 of the CSVd isolate, and sense primer (5'-ATC CCC GGG GAA ACC TGG AGG AAG T-3') was homologous to CSVd nucleotides 88 to112 of the isolate (Genbank accession no. AJ001850). RT-PCR was carried out using GeneAmp RNA PCR kit (Perkin Elmer Cetus Inc., Norwalk, CT). Two (2) micrograms of nucleic acids were added to an annealing reaction mixture containing 2 µl of 10X PCR buffer (100 mM Tris-HCl, pH 8.3, 50 mM KCl), 5 mM of $MgCl_2$, 1 mM of dNTPs each, 1 µl of RNase inhibitor (1 U/µl), 1 µl of MuLV reverse transcriptase (2.5 U/µl), and 100 pmol CSVd antisense primer to a final volume of 20 µl for reverse transcription. The mixture was treated at 42°C for 15 min and denatured for 5 min at 99°C, and then chilled at 5°C for 5 min according to the manufactures instruction (GeneAmp RNA PCR kit). Eighty (80) microliters of the PCR mixture containing 8 µl of 10X PCR buffer, 2 mM $MgCl_2$, 0.5 µl of AmpliTaq DNA polymerase (2.5 U/100 µl; Perkin Elmer Cetus Inc., Norwalk, CT), and 1 µl of 100 pmol sense CSVd primer were added to each reaction tube for PCR with the following cycles: denaturation at 94°C for 30 sec, primer annealing at 53°C for 1 min, and extension at 72°C for 1 min for 40 cycles with a final extension at 72°C for 7 min.

Analysis of RT-PCR amplified products. RT-PCR amplified products were analyzed on 2% agarose gel in 0.5X TBE buffer (44.5 mM Tris-HCl, 44.5 mM boric acid, and 1 mM Na_2-EDTA, pH 8.3) at 100 volt for 30 min. Molecular weight marker used was ϕX174-*Hinc*II. PCR products from J2 CSVd (shiwaku, 1996a) isolate were used as control. Nucleic acids of the gels were stained with ethidium bromide.

Cloning of RT-PCR products for CSVd DNA. Two isolates of amplified 354 bp CSVd PCR products were eluted from the agarose gel using the Promega GeneClean III kit (Promega, Madison, WI, USA). Eluted PCR products were cloned into pGEM-T easy vector (Promega). Recombinants were screened for white and antibiotic resistant colonies on LB media containing ampicillin, X-Gal, and IPTG. Plasmid DNA was extracted by alkaline lysis methods (Sambrook et al., 1989).

Nucleotide sequence. The nucleotide sequences of the cloned PCR products for two isolates were determined using ABI Prism™ Terminator Cycle Sequencing Ready Reaction Kit and ABI Prism 377 Genetic Analyzer (Perkin Elmer, USA). Nucleotide sequence analysis was carried out using DNASTAR program. The phylogenetic tree analysis derived from the comparison of nucleotide sequences was constructed using DNASTAR program.

Results

Bioassays. Chrysanthemum cv. Mistletoe grafted on naturally CSVd K1 (Fig. 1) and K2 (Fig. 2) infected chrysanthemum cv. Chunkwang produced yellow spots on the leaves 2 months after grafting (Fig. 3). *G. aurantiaca* inoculated mechanically with the sap of naturally CSVd infected chrysanthemum cv. Chunkwang did not produce any symptom. However, viroid was detected by RT-PCR (data not shown). Symptoms of leaf distortion or mottle on leaves were induced in *L. esculentum* cv. Rutgers (Fig. 4).

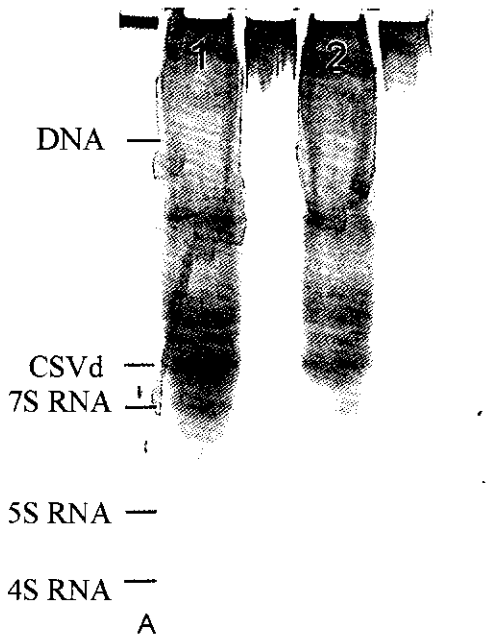
Extraction and purification of nucleic acids. Host DNA, 7S RNA, 5S RNA, and 4S RNA were shown equally (Fig. 5A) from the total nucleic acids of CSVd K1 and K2 infected chrysanthemums. The infected chrysanthemum extract contained an extra band not found in the healthy plant (Fig. 5B). The absorbance of total nucleic acids from the stunted chrysanthemum had the typical ultraviolet spectrum of a maximum of 260 nm and a minimum of 232 nm (Fig. 6). The yield of the total nucleic acid was 48.6 mg/200 g of CSVd infected chrysanthemum leaves.

RT-PCR. A DNA product of 354 bp (full-length) was detected after amplification of nucleic acids from CSVd infected chrysanthemum cv. Chunkwang and from J2 isolate (Fig. 7). This product was not detected in the total nucleic acids from healthy chrysanthemum leaves (Fig. 7).

Nucleotide sequence and phylogenetic analysis. The nucleotide sequence of K1 and K2 isolates were determined and submitted to the Genbank under the accession numbers AF394452 and AF394453, respectively. The number of nucleotide sequence of the two isolates was 354 (Fig. 8). Two nucleotides and one nucleotide were lacking compared with At and Am2 isolates, respectively (Fig. 8). Nucleotide sequence of K1 isolate differed from that of K2 isolate. Compared with K1 isolate, K2 showed substitutions T → C in position 26 and C → T in position 296 (Fig. 8). The nucleotide sequence of K1 was the same as that of J2 isolate (Shiwaku, 1996b) (Fig. 8). Phylogenetic tree analysis derived from the comparison of nucleotide sequences using Clustal method (DNASTAR program) is shown in Fig. 9. Phylogenetic tree analysis of nucleotide sequence of CSVd isolates indicated that K1 was closely related with J2 and Am2 isolates.

Discussion

Chrysanthemum stunt viroid (CSVd) (Mayo, 1999) was first observed in 1945. Nowadays, the pathogen occurs prevalently worldwide (Lawson, 1987) and causes serious losses in chrysanthemums. In spite of the worldwide distribution of CSVd in chrysanthemum and its effect on the quality of the plant, there has been no research on CSVd in



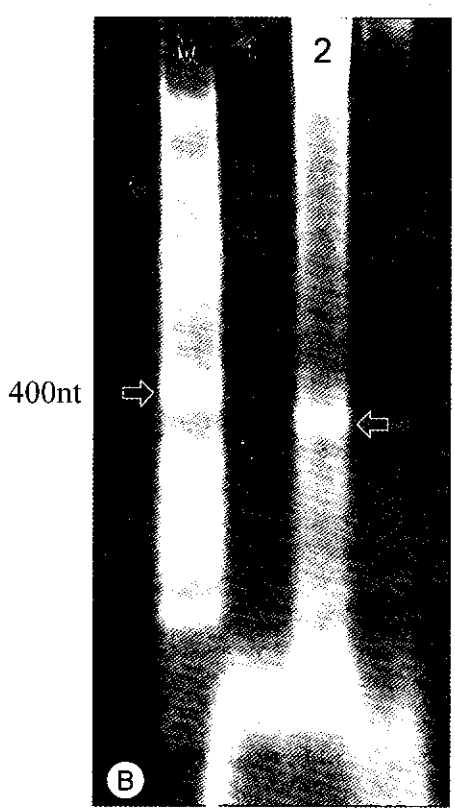


Fig. 5. Polyacrylamide gel electrophoresis under non-denaturing conditions of necleic acids from *Chrysanthemum stunt viroid*-K1- and -K2-infected chrysanthemum (**A** lane 1,K1; lane 2, K2; B lane 2, K1) and healthy plant (**B**, lane 1). M: Perfect RNA™ marker (Novagen, Madison, WI). Nucleic acids was run on 5% acrylamide, 0.25% methylene bisacrylamide gels (14 × 14 × 0.3 cm) in the 40 mM tris-acetate, pH 8.1, 20 mM sodium acetate, and 2 mM EDTA buffer at the electric fields of 10 mA/gel for 13 hours. Nucleic acids were stained with silver (**A**) and ethidium bromide (**B**).

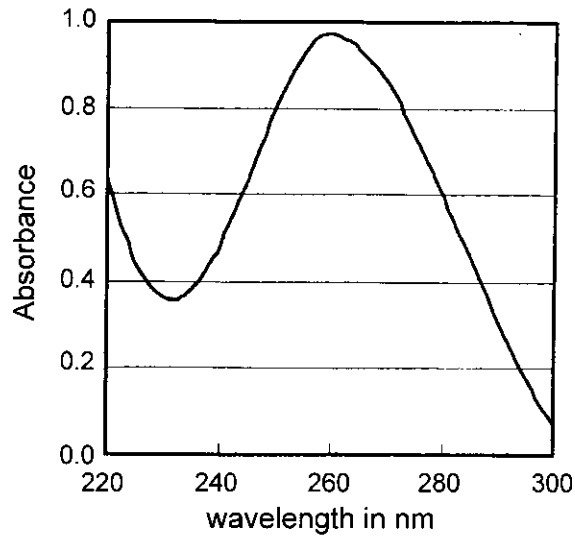


Fig. 6. UV absorption spectrum of the total nucleic acids from *Chrysanthemum stunt viroid*-infected chrysanthemum.

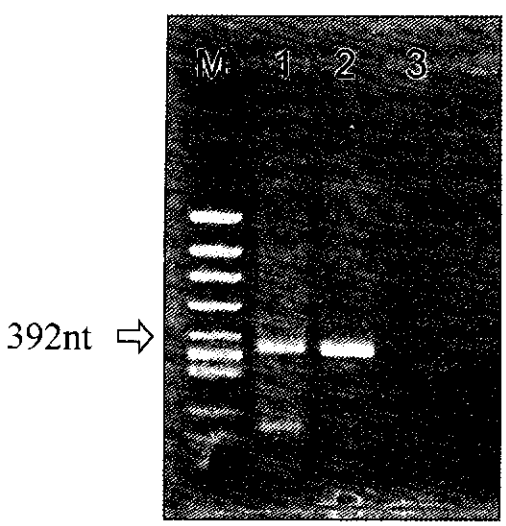


Fig. 7. Agarose gel electrophoresis of PCR products produced from *Chrysanthemum stunt viroid* (CSVd)-J2 isolate (lane 1), CSVd-K1-infected chrysanthemum (lane 2) and healthy plant (lane 3). M: DNA size marker ΦX174-*Hinc*II. Nucleic acids were stained with ethidium bromide.

Korea. Two CSVd isolates, K1 and K2, were identified in field-grown chrysanthemums based on symptomatology, bioassay, RT-PCR, and nucleotide sequence determination.

In this study, K1 and K2 were shown to be CSVd isolates based on the following: 1) typical CSVd disease symptom of stunt; 2) symptom development on chrysanthemum cv. Mistletoe; 3) amplification of cDNA from chrysanthemums showing disease symptoms using CSVd specific primers; and 4) nucleotide sequences of K1 and K2. These results indicated that chrysanthemums showing stunt with leaf distortion (K1) or stunt with chlorosis of leaves and mottle on

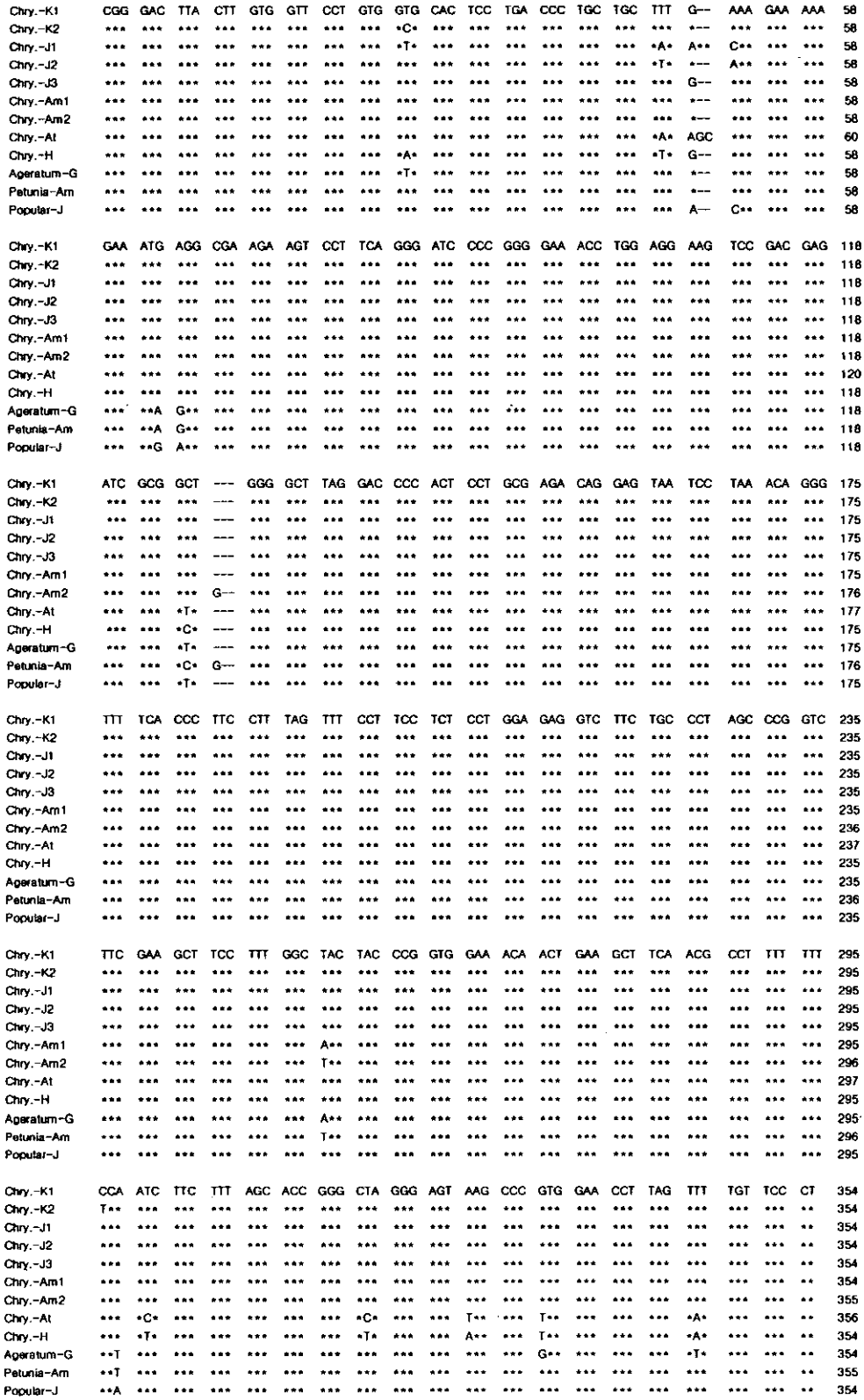


Fig. 8. Alignment of the nucleotide sequences of *Chrysanthemum stunt viroid* isolates. K1, GenBank accession no. AF394452; K2, AF394453; J1, D88895; J2, Shiwaku, 1996b; J3, AB055972; Am1, AJ001849; Am2, AJ001852; At, M19505; H, AJ000046; Ageratum (G), Z68201; Petunia(Am), U82445; Popular(J), AB006737.

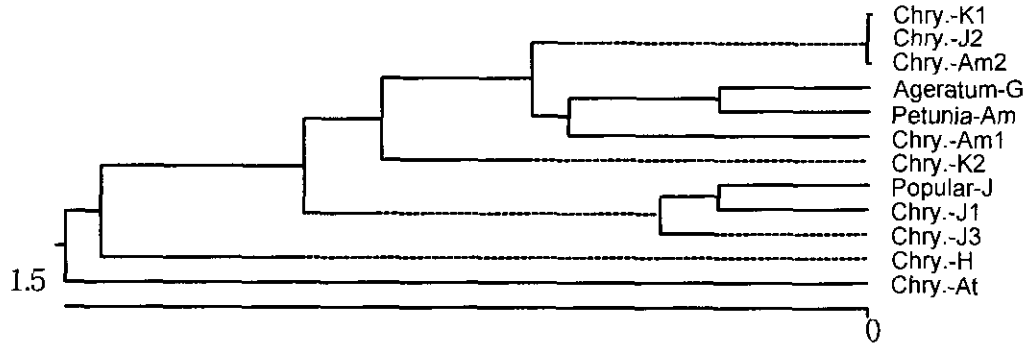


Fig. 9. Phylogenetic tree analysis of the nucleotide sequences of CSVd isolates. The relative branch length of this dendrogram corresponds to the average divergence between the different isolates. Dotted lines indicate a negative branch length. The phylogenetic tree was constructed using Clustal method (DNASTAR program).

leaves (K2) were caused by CSVd infection. Generally observed viroid infection symptoms in field-grown chrysanthemums were stunt with leaf distortion. Symptom of chlorosis of leaves and mottle on leaves was uncommon, although yellowing of young leaves of chrysanthemum was previously reported (Dusi et al., 1990). Reaction of chrysanthemum cv. Mistletoe grafted on naturally CSVd-K1-infected and -K2-infected cv. Chunkwang showed that these chrysanthemums were infected with CSVd previously reported on chrysanthemum (Dusi et al., 1990; Olson, 1949; Shiwaku et al., 1996a). As Olson (1949) reported, chrysanthemum cv. Mistletoe was a sensitive plant for the diagnosis of CSVd by showing yellows spots on leaves. The reactions of tested plants, *L. esculentum* and *G. aurantiaca*, inoculated with CSVd showed some differences compared with a previous report. Dusi et al. (1990) reported that *L. esculentum* inoculated with CSVd induced interveinal chlorosis or leaf curling. However, in this study, interveinal chlorosis mottle on leaves was induced. Similar symptoms developed in *G. aurantiaca*.

To exclude viral RNA infecting chrysanthemums, *Chrysanthemum virus* B or *Tomato aspermy virus*, total nucleic acids were extracted from CSVd-K1 or -K2 infected chrysanthemum seedlings. Total nucleic acids from K1 infected chrysanthemums contained an extra band, which was thought to be associated with CSVd RNA not found in healthy plants. These band patterns were similar to the reports of Palukaitis and Symons (1980).

Six chrysanthemum samples were collected from different farms in different areas for the determination of nucleotide sequences. Five of six samples showed similar nucleotide sequence as K1 (data not shown). Based on the results, CSVd-K1 appeared to be the most common isolate in chrysanthemum cv. Chunkwang in Korea. Phylogenetic tree analysis of nucleotide sequence of CSVd isolates indicated that K2 was a little distantly related with K1. Among the isolates tested, the most closely related with K2 were K1, J2, and Am2. K2 isolate was assumed to result from a mutation of K1. Results of this study showed that K1 isolate might be imported from Japan through chrysanthemum plant cuttings, and that K2 is a new variant of CSVd.

Up to now, only chrysanthemum cv. Chunkwang was known to be naturally infected with CSVd in the fields. And since this cultivar is the most commonly grown in Korea, further studies are needed to protect chrysanthemums from CSVd infection.

Acknowledgments

The authors gratefully acknowledge Dr. Kuniko Shiwaku (Hyogo Prefectural Agricultural Institute, Befu, Kasai, Japan) for providing the CSVd RNA Japanese isolate (J2) used in this study.

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