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Occurrence of *Chrysanthemum chlorotic mottle viroid* in Chrysanthemum (*Dendranthema grandiflorum*) in Korea

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Chrysanthemum chlorotic mottle viroid **(CChMVd) isolates have been identified from chrysanthemum showing yellow spots or infected without symptom. They were 399-400 nucleotides length of RNA. CChMVd-SSHA6 (GenBank accession no. DQ450682) revealed a GAAA to UUUC substitution in positions 82-85 of CChMVd-MSIN34 (GenBank accession no. DQ402041).** ***In vitro*** **RNA transcripts with the complete CChMVd sequence were infectious and induced the typical CChMVd infection symptom of yellow spots in chrysanthemum cv. Sharotte. CChMVd caused reduction in growth in some cultivars, whereas some cultivars were not affected. This is the first report on the occurrence of CChMVd in chrysanthemum in Korea.**

Keywords **:** *Chrysanthemum chlorotic mottle viroid*, Chrysanthemum, viroid

Viroid, small single-stranded circular RNAs, (between 246 and 401 nt) is able to infect certain plants. Viroids do not code for any protein, their pathogenic effects must result from direct interaction of the viroid RNA itself, or of some of its replicative intermediates, with one or more host components (Diener et al., 1993). The viroid species sequenced so far have been grouped within two families (Matthews, 1991).

Two kinds of viroids were reported in chrysanthemum world-wide (Bouwen and Zaayen, 1995). These are *Chrysanthemum stunt viroid* (CSVd; Chung et al., 2005; Diener and Lawson, 1973; Dusi et al., 1990, 1995; Hooftman et al., 1996; Kusunoki, 1993; Shiwaku, 1996) and *Chrysanthemum chlorotic mottle viroid* (Diener et al., 1977; McGovern et al., 1988; Navarro and Flores, 1997; Peña et al., 1999). *Chrysanthemum chlorotic mottle viroid* (CChMVd) was first described by Dimock and Geissinger (1969). CChMVd has been reported to occur in infected chrysanthemums in Denmark (Paludan, 1980), France (Monsion et al., 1980) and India (Singh et al., 1978).

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Symptoms caused by CChMVd in chrysanthemum have been reported as yellow-green mottling and eventually developed pronounced general chlorosis and were somewhat dwarfed in some cultivars including 'Yellow Delaware' (Dimock et al., 1971). 'Knob Hill' exhibited chlorotic spots, vein clearing and mild chlorosis, whereas in some cultivars although infected, remained symptomless (Dimock et al., 1971). Additional symptoms include dwarfing of leaves, flowers, and the entire plant and delay in flower development (Dimock et al., 1971). Sap inoculation, grafts and tissue implantation transmit the pathogen. No insect vector is known (Horst, 1987).

CChMVd poses a potentially serious threat to the chrysanthemum industry (Horst, 1987). In this report, full length nucleotide sequences of CChMVd isolated from field-grown chrysanthemum were determined, and the effect of CChMVd on the growth of chrysanthemum was described. This is the first report on the occurrence of CChMVd in chrysanthemum in Korea.

Materials and Methods

Preparation of RNA. RNA was prepared from chrysanthemum cvs. Sharotte and Kasandra showing yellow spots (Fig. 1), and cv. Sinma infected without symptom (Fig. 2) with 0.1 g of leaves using CF11 cellulose according to a method described previously (Shiwaku et al., 1996). Pellet was resuspended in 50 μl of nuclease-free water.

Primers and RT-PCR conditions. Two pairs of primer (CChMVd1F/CChMVd1R; CChMVd2F/CChMVd2R) were designed. Primer pair CChMVd1F/CChMVd1F was designed on the basis of CChMVd (GenBank accession no. AJ247114). The sequence of forward primer (CChMVd1F) was homologous to nucleotides 49~67 of the CChMVd and that of reverse primer (CChMVd1R) was complementary to nucleotides 29~48 of the CChMVd. The sequence of the primer region of CChMVd1F/CChMVd1R was confirmed with a second primer pair (CChMVd2F/CChMVd2R). The sequence of forward primer (CChMVd2F) was homologous to nucleotides 121~140 of DQ450682 (GenBank

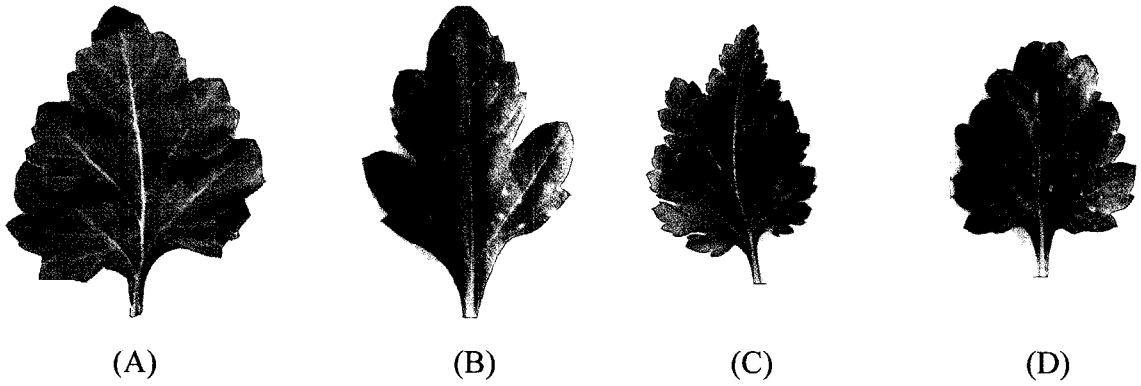

Fig. 1. Symptoms induced by field-infection of *Chrysanthemum chlorotic mottle viroid* (CChMVd) in chrysanthemum cv. Sharotte. (A) Healthy plants; (B) CChMVd; (C) *Chrysanthemum stunt viroid* (CSVd); (D) double infection with CChMVd and CSVd.

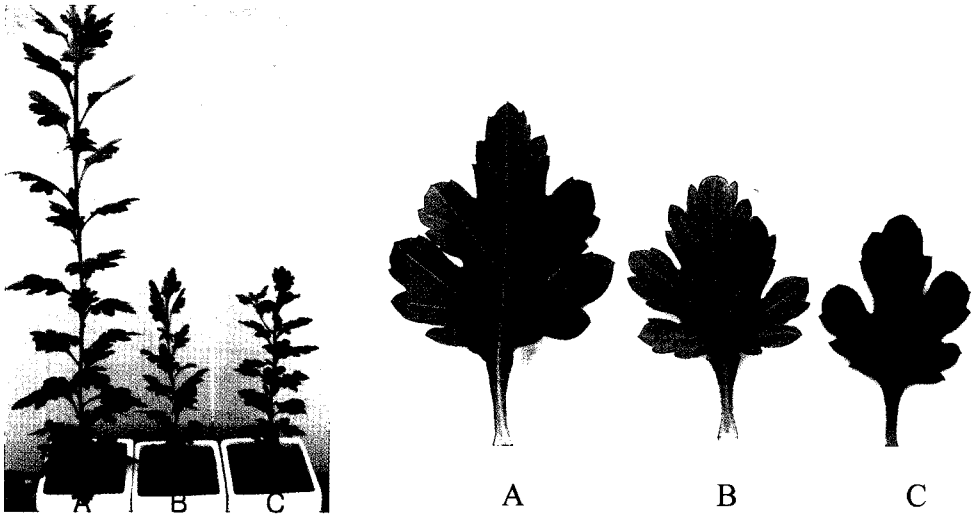

Fig. 2. Field-infection of chrysanthemum cv. Sinma with CChMVd. (A) Healthy plants; (B) CSVd single infection; (C) double infection with CChMVd and CSVd.

accession no.) and that of reverse primer (CChMVd2R) was complementary to nucleotides 101~120 of DQ450682. Complementary DNA (cDNA) synthesis was accomplished as follows: One μl of RNA and 1 μl of 10 pmole reverse primer was heated at 70°C for 5 min followed by adding 1X Reaction buffer, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 1 μl of AMV reverse transcriptase (Promega, USA) and 1 μl of RNase inhibitor (1 U/μl) on ice, and incubated at 37°C for 1 hr. PCR amplification was performed in 50 μl containing 5 μl of the cDNA solution, 0.2 mM of each dNTP, 2 mM $MgCl_2$, 10 pmole of each primer, 2.5 units of DNA polymerase (Promega, USA), and the 1X PCR buffer. Forty PCR cycles were conducted in PTC-0220 Perlitier Thermal Cycler (MJ Research, MA, USA). The thermal conditions were as follows: denaturation at 94°C for 30 sec (2 min for the first cycle), annealing at 48°C for 1 min and extension at 72°C for 1 min.

Determination of nucleotide sequences. The amplified PCR products of the expected full length were eluted and cloned in the pGEM-Teasy vector. The ligation mixture was used to transform competent cells of *Escherichia coli* JM109. Nucleotide sequences of the cloned PCR products were determined using ABI Prism™ Terminator Cycle Sequencing Ready Reaction Kit and ABI Prism 377 Genetic Analyzer (Perkin Elmer, USA).

Preparation of RNA transcripts and inoculation. To compare the pathogenicity of CChMVd isolates SSHA2, SSHA6 and MSIN34, cv. Sharotte plants infected with each CChMVd isolate were prepared by inoculation with 20 μl of RNA transcripts per plants after wounding stems using blazer. Five chrysanthemum plants were replicated for each isolate for the determination of growth. Transcripts were generated by *in vitro* transcription of a plasmid DNA

containing a full length SSHA2, SSHA6 or MSIN34 cDNA insert. Template plasmid DNA was linealized by digestion with *Sal* I restriction enzyme followed by phenol extraction. RNA transcripts were prepared using mMESSAGE mMACHINE T7 according to manufactures' instruction (Ambion, USA).

Graft inoculation with CChMVd-infected chrysanthemum. Healthy chrysanthemum cultivars Sinma, Kasandra, Argus, Chunkwang and Mistletoe were grafted on naturally CChMVd-infected cv. Sharotte. CChMVd-infected cv. Sharotte plants were prepared by cutting and used for grafting stock. Six weeks after grafting RNA was prepared from leaves of scion plants and determined CChMVd infection by RT-PCR. CChMVd-infected scion plants were propagated by cutting and planted 5 plants per cultivar, and measured plant height and leaf length at 2 months after planting.

Results

Disease symptoms and damages. Naturally CChMVd-infected chrysanthemum cv. Sharotte revealed yellow spots on leaves and mixed infection with *Chrysanthemum stunt viroid* (CSVd) induced additional symptom of vein clearing along with reduced leaf size (Fig. 1). Cultivar Sinma spontaneously infected with CSVd alone or mixed infection with CChMVd showed no symptoms on leaves, however CChMVd caused slight reduction in leaf size than CSVd single infection (Fig. 2).

Chrysanthemum cultivars Kasandra, Argus, Mistletoe were affected significantly in plant height and leaf length at 3.5 months after graft-inoculation with CChMVd (Table 1).

Table 1. Effect of *Chrysanthemum chlorotic mottle viroid* on the growth of chrysanthemum cultivars

Cultivar	Division	Plant height (cm)	Leaf length (cm)
Sharotte	Healthy	33	6.1
	CChMVd	31 ns[a]	6.0 ns
Sinma	Healthy	42	8.3
	CChMVd	42 ns	7.8*
Kasandra	Healthy	42	8.0
	CChMVd	38*	7.3*
Argus	Healthy	29	9.2
	CChMVd	22**	8.3**
Chunkwang	Healthy	42	7.8
	CChMVd	42 ns	7.8 ns
Mistletoe	Healthy	26	8.2
	CChMVd	19**	6.7**

[a]T-test: ns=nonsignificant, *significant P=0.05, **significant P=0.01

Plant height and leaf length was reduced by 10~27% and 6~18% compared with healthy chrysanthemum, respectively. However cvs. Sharotte and Chunkwang were not affected by CChMVd in growth. CChMVd caused reduction in only leaf length in cv. Sinma (Table 1). Mistletoe induced numerous yellow spots with CChMVd at 6 weeks after graft inoculation (Fig. 3).

Nucleotide sequences. Full-length nucleotide sequences of 5 CChMVd isolates were determined (Fig. 4). They consisted of 399~400 nt. MSIN34 was isolated from cv. Sinma; SHA35, SSHA2 and SSHA6 were isolated from cv. Sharotte; and SKA44 was isolated from cv. Kasandra. Isolates SSHA2, SSHA6 or SKA44 revealed a GAAA to UUUC substitution in positions 82-85 of MSIN34 or MSHA35. Nucleotide sequences of ChMVd-MSIN34 and CChMVd-SSHA6 were submitted to the GenBank under the accession number of DQ402041 and DQ450682, respectively.

Infectivity of *in vitro* RNA transcripts and pathogenicity of CChMVd isolates. Cultivar Sharotte became infected and developed symptoms of yellow spots by infection with *in vitro* RNA transcripts (Fig. 5). Sharotte infected with CChMVd-SSHA2, -SSHA6 or –MSIN34 did not show difference in symptom and in plant height (Table 2). However, leaf size of nonsymptomatic isolate MSIN34 was significantly larger than symptomatic isolates SSHA2 or SSHA6.

Discussion

CChMVd caused reduction of growth by 10~27% in 3 out of 6 chrysanthemum cultivars tested, but the other cultivars were not affected. It caused slight reduction in growth than CSVd which caused 30~50% reduction in most chrysanthemum cultivars (Chung et al., 2005). This result was consistent with Paludan (1980).

Cultivar Mistletoe exhibited yellow spots within 6 weeks under 25~30°C green-house condition, and may be used for bioassays (Fig. 3). It induced numerous yellow spots at 2 months after inoculation. CSVd has been known to cause yellow spots in some cultivars (Chung et al., 2005). CChMVd also induced yellows spots in cvs. Sharotte, Kasandra (picture not shown) and Mistletoe in this study. Cultivar Sharotte revealed yellow spots by infection with either viroid (Chung et al., 2005).

Romaine and Horst (1975) reported the viroid nature of CChMVd and suggested that the narrow host range and the less contagious characteristics as compared with other viroids were due to instability of the agent in crude extracts. Likewise, numerous CChMVd isolates were existed in

Fig. 3. Yellows spots on chrysanthemum cv. Mistletoe induced by graft inoculation on naturally CChMVd-infected chrysanthemum.

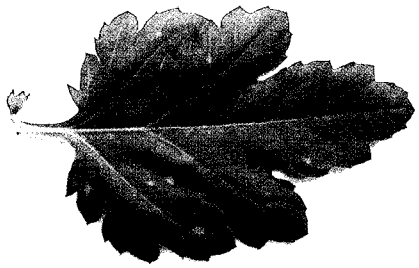

Fig. 5. Yellows spots induced by mechanical inoculation with *in vitro* RNA transcripts of CChMVd isolate CChMVd MSIN34 in chrysanthemum cv. 'Sharotte'.

Table 2. Comparison of the growth of chrysanthemum cv. Sharotte infected with different CChMVd isolates

Isolates	Plant height (cm)	Leaf length (cm)
CChMVd-SSHA2	32.5 a[z]	9.2 b
CChMVd-SSHA6	31.0 a	8.9 b
CChMVd-MSIN34	32.0 a	10.0 a

[z]Mean separation within each columns by Duncan's multiple range test at 5% level.

field-infected chrysanthemum in this study. The result was consistent with Peña et al. (1999): twelve variants were isolated from cv. Bonnie Jean after inoculation with CChMVd-NS strain. Peña et al. (1999) proposed that substitution of UUUC → GAAA in position 82~85 was sufficient to change the symptomatic phenotype into the nonsymptomatic one. In this study, CChMVd-MSIN34 and -MSHA35 were grouped to nonsymptomatic isolate, and SSHA2, SSHA6 and SKA44 were grouped to symptomatic isolate based on the suggestion by Peña et al. (1999). Chutivar Sharotte revealing vein clearing symptom (Fig. 1)

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                    20                  40                  60                  80                 100
MSIN 34 GGCACCUGACGUCGGUGUCCUGACGAAGAUCCAUGACAGGAUCGAAACCUCUUCCAGUUUCGGCUUGUGCGGGAGUAAAUCGAAAGAUCUCUCCACAGCC
MSHA 35 ....................................................................................................
SSHA  6 ..................C.................................................GCUUUCA.....................
SSHA  2 ..................C.................................................GCUUUCG.....................
SKA  44 ..........................................A.....................GCUUUCA.....................

                   120                 140                 160                 180                 200
MSIN 34 UCGUCAGGAAACCCACUUCAGGUCUCGACUGGAAGGUCGUAAAACUUCCUCUCUAAGCGGAGUAGAGGUAAAUACCUCCGUCCCAUCCCGGGAGGAAAGG
MSHA 35 ....................................................................................................
SSHA  6 ..................................U........C.............................................................
SSHA  2 ..................................U..-.....C.....................................C.....................
SKA  44 ..................................U........C.....................................C.....................

                   220                 240                 260                 280                 300
MSIN 34 GAUGGGGACCCGGAACAGACCCAGUUCCGGUCCUUUGGAGUCCAUUUUUCUCGUUGGAUAUUCUCCUCGGAGAAGGGAAAUGGGGCCAGUCCCAGUCGGU
MSHA 35 ....................................................................................................
SSHA  6 ....................................................................................................
SSHA  2 .............G......U..C.....................C..C....A.........U....G.......G.....................
SKA  44 ..........................................C..C.....................G.......G.....................

                   320                 340                 360                 380                 400
MSIN 34 UCGCUCUCGUAGUCACAGCCGCUGGGGAACCUAGGCAGAAGGCUGGACGGAGTCUUAGUCCACUCCAAAGGACCUUGGGUUUAGAAACCCCCAAGAGGUC
MSHA 35 ....................................................................................U...............
SSHA  6 ....................................................................................G.......
SSHA  2 ..................................U.....................................................................
SKA  44 ..................................U.....................................CU..-.....................
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Fig. 4. Sequence alignment of five isolates from chrysanthemum cvs. Sinma, Sharotte and Kasandra infected with *Chrysanthemum chlorotic mottle viroid* naturally. Dots indicate residues identical to the MSIN34 sequence and dashes denote gaps.

consisted of two types of isolates: symptomatic isolates SSHA2 and SSHA6 and a nonsymptomatic isolate MSHA35. The coexistence of sequence variants with different pathogenicity within a viroid strain has also been reported in *Peach latent mosaic viroid* (Ambrós et al., 1998).

Meanwhile, in this study two symptomatic isolates SSHA2 and SSHA6, and a nonsymptomatic isolate MSIN34 did not show difference in symptom and in plant height (Table 2). Only leaf size of nonsymptomatic isolate MSIN34 was significantly larger than symptomatic isolates SSHA2 or SSHA6. This result was not consistent with the report of Peña et al. (1999). According to the report, symptomatic isolates SSHA2 or SSHA6 should reveal more severe symptom than nonsymptomatic isolate MSIN34. We presumed from the result that responses by symptomatic or nonsymptomatic isolates might be dependent on cultivar, because cv. Sharotte was not damageable cultivar (Table 1). More studies were needed to prove the difference in pathogenicity between symptomatic and nonsymptomatic isolates grouped based on the sequence substitution in position 82~85.

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