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Occurrence of *Tomato spotted wilt virus* in Chrysanthemum (*Dendranthema grandiflorum*) in Korea

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***Tomato spotted wilt virus* (TSWV) has been identified in commercial chrysanthemum cultivars in Korea. Nucleotide sequences of the N gene of TSWV-ch14 isolated from infected chrysanthemum were determined and deposited in GenBank under accession no. DQ453158. The symptoms consisted of dark colored leaf necrosis, black streaks along the stem, wilting of plant parts in 'Sinma'; and chlorotic spots, necrosis of axillary shoots and withering of leaves in 'Hwarang'. Electron micrographs of leaf preparation of *Nicotiana rustica* infected with TSWV-ch14 contained spherical particles around 85 nm in diameter. TSWV was identified from chrysanthemum by sequence determination of N nucleocapsid protein and virion observation by transmission electron microscope. This is the first reported observation on TSWV in chrysanthemum in Korea.**

Keywords : Chrysanthemum, TSWV

Four kinds of viruses and two viroids have been reported as major viral disease on chrysanthemum (Bouwen and Annemarie, 1995) worldwide. These are *Tomato aspermy virus* (TAV), *Chrysanthemum virus* B (CVB), *Tomato spotted wilt virus* (TSWV), *Impatiens necrotic spot virus* (INSV), *Chrysanthemum stunt viroid* (CSVd) and *Chrysanthemum chlorotic mottle viroid* (CChMVd). TSWV and INSV are two tospoviruses infecting chrysanthemum (Daughtrey et al., 1997; Verhoeven et al., 1996).

TSWV causes symptoms of chlorotic spots, foliar necrosis, leaf bronzing, chlorotic ring, line patterns, internal necrosis, stem cankers and stunting (Sherman et al., 1998; Verhoeven et al., 1996). Affected plants often die. Commercial flower production is also affected by losses in flower weight and petal number, and reduced numbers of flowers in spray type (Horst and Nelson, 1997).

The primary movement of TSWV from one plant to another has been known by feeding of thrips: Nine species are reported as vectors of TSWV (Gibbs, 1983). Western flower thrips (*Frankliniella occidentalis*) is believed to be a major reason for the TSWV transmission. Chrysanthemum is one of ornamental crops devastated by TSWV. As Western flower thrips spread, TSWV has been epidemic in chrysanthemum in USA (Hausbecak et al., 1992), Canada (Allen and Broadbent, 1986), Japan (Tsuda et al., 1994) and European countries including Greece (Chatzivassiliou et al., 1996), Portugal (Louro, 1996) and the Netherland (Verhoeven et al., 1996).

TSWV was epidemic in commercial chrysanthemum fields cultivating cultivar Sinma in Taean, Chungnam Province. Infected plants showed chlorotic or necrotic spots on leaves, and stem necrosis with withering of plant parts. The first symptoms were found in unknown pot-mum cultivar in Ansan, Gyeonggi Province in 2005. By now TSWV has spread over the commercial chrysanthemum fields in Taean and Yesan, Chungnam Province.

In this study TSWV has been identified from commercial chrysanthemum crops showing necrotic and chlorotic leaf symptoms by sequence determination of N nucleocapsid protein and virion observation by transmission electron microscope (TEM). This is the first reported observation on TSWV in chrysanthemum in Korea.

Materials and Methods

Plant material. Chrysanthemum cultivars 'Sinma' and 'Hwarang' showing symptoms of leaf withering or chlorotic spots were collected from commercial green-houses in Taean and Yesan, Chungnam Province in 2006. Those plants were maintained in glass-house and used for the determination of nucleotide sequences and for inoculum of *Nicotiana rustica.*

Preparation of RNA. RNA was prepared with 0.1 g of chrysanthemum leaves using RNeasy plant mini kit (Qiagen GmbH, Hilden, Germany) according to manufacture's instruction.

Primers and RT-PCR conditions. A pair of primer was designed on the basis of the N gene of a TSWV strain

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(GenBank accession no. AB175809). The sequence of forward primer was homologous to nucleotides 1-22 (ATGTCTAAGGTTAAGCTCAC) of the TSWV strain and that of reverse primer was complementary to nucleotides 758-777 (TTAAGCAAGTTCTGCGAGTT) of the TSWV strain. Complementary DNA (cDNA) synthesis was accomplished as follows: Mixture of 1 μl of RNA and 1 μl of 10 pm reverse primer was heated at 70°C for 5 min followed by adding 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 2.5 mM $MgCl_2$, 1 mM each dNTP, 1 μl

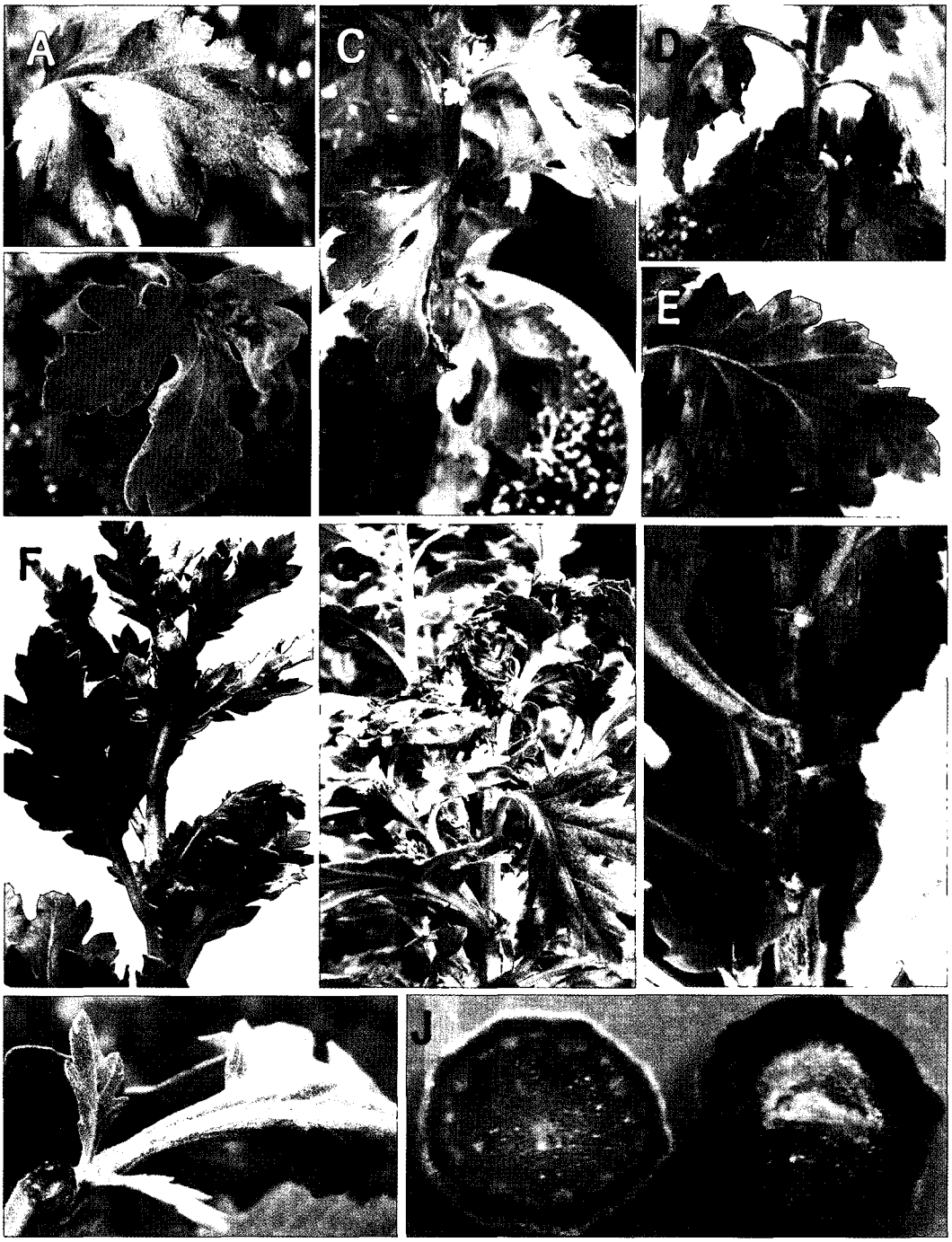

Fig. 1. Symptoms observed from chrysanthemum cvs. Hwarang and Sinma naturally infected with *Tomato spotted wilt virus*-ch 14. A~D: Hwarang; E~J: Sinma; A: chlorotic spots; B: vein lesions on underside of leaf; C: leaf necrosis along with necrosis of axially shoots; D: leaf withering; E: leaf bronzing; F: dark colored leaf necrosis; G: withering of plant parts; H: brown to black streaks along the stem; I: stem necrosis accompanying the leaf necrosis; J: healthy (left) and discolored vascular bundle and hollow in stem pith (right).

of reverse transcriptase (Promega, USA) and 1 μl of RNase inhibitor (1 U/μl) on ice and incubated at 42°C for 1 hr. PCR amplification was performed in 50 μl containing 5 μl of the cDNA solution, 1 mM each dNTP, 2 mM $MgCl_2$, 10 pm each of primer, 2.5 units of DNA polymerase (Promega, USA), and the 10× 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.

To prove chrysanthemum plants used for determination of symptoms were not infected with other viral or viroid disease except TSWV, RT-PCR was conducted with specific primers. Test for TAV, CVB and CSVd was conducted as described previously (Chung, 2002; Chung et al., 2005). For diagnosis of CChMVd and INSV, two pairs of primer were designed based on GenBank accession no. AJ247114 and AB1099100, respectively: For INSV forward primer was GTAGCATTAAACATGCTGTAAATG and reverse primer was GTCAAGCTTTTG ACTCAATCTGAT; for CChMVd forward primer was CTCTTCCAGTTTCGGC-TTG and reverse primer was GTTTCGATCCTGTCAT-GGAT.

Determination of nucleotide sequences. The amplified PCR products of 777 bp were eluted and cloned in the pGEM-T easy vector (Promega, Madison, WI, USA). 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).

Sequences were aligned using a program DNASTAR software version 7 (Madison, WI, USA). Sequences of a clone (designated as TSWV-ch14) were compared with other TSWV strains registered in GenBank (www.ncbi.nlm.nih.gov).

Observation of TSWV particles by TEM. *N. rustica* was rubbed with crude sap extracted from chrysanthemum 'Hwarang' showing chlorotic symptom. Ten days post inoculation, upper leaves showing systemic symptom were collected. Crude sap extracted from the upper leaf was mixed with 0.5% phosphotungustic acid and was observed using Carl Zeiss LEO 906 TEM.

Results

Sympomatology. Chrysanthemum plants used for the present study were not infected with other viral diseases of TAV, CVB, CSVd, CChMVd or INSV. Cultivar 'Hwarang' naturally infected with TSWV showed two different leaf symptoms: Chlorotic spots (Fig. 1A) and vein lesions on underside of leaf (Fig. 1B). Those symptoms advanced to axially shoots and upper leaves (Fig. 1C) resulting in wilting of leaves (Fig. 1D). In cultivar 'Sinma', symptoms consisted of chlorotic to necrotic spots, leaf bronzing (Fig. 1E) and black colored leaf necrosis (Fig. 1F). Occasionally, withering of plant parts (Fig. 1G), brown to black streaks along the stem (Fig. 1H), stem necrosis accompanying the leaf necrosis (Fig. 1I) were observed. Cross section of the stem of Fig. 1I revealed discolored vascular bundle and hollow (Fig. 1J).

In the later growth stage of 'Sinma' infected with TSWV, flower stem became withered (Fig. 2A) and sometimes necrosis was found in the center of flowers (Fig. 2B).

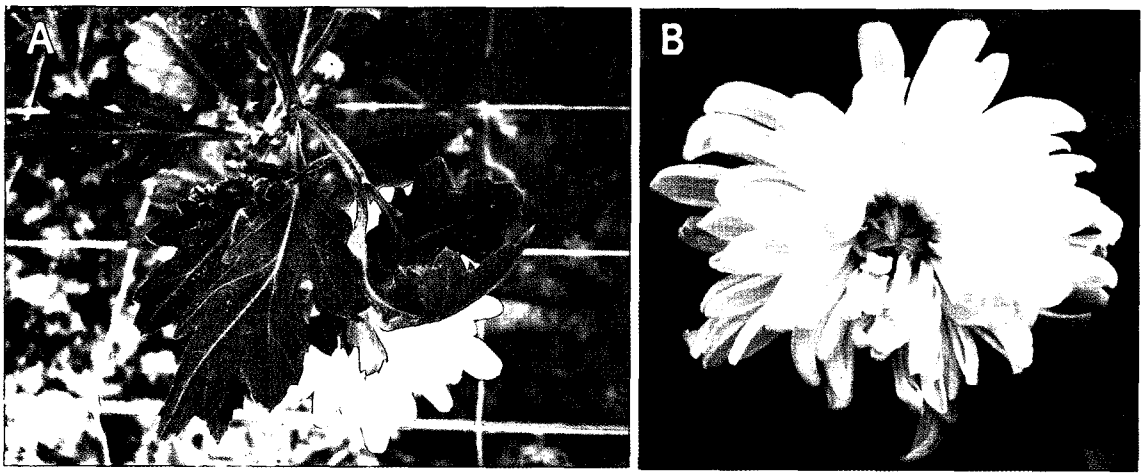

Fig. 2. Damages on flowers caused by TSWV in chrysanthemum cultivar Sinma. (A) Flower stem necrosis; (B) necrosis in the center of flower.

Table 1. Sequence homology percent of TSWV-ch14 with other TSWV strains registered in GenBank (www.ncbi.nlm.nih.gov)

	AY848922	AY744471	AB190819	AB175809	AB038341	AB010997
TSWV-ch14	97.7	96.9	97.3	96.7	97.6	98.1
AY848922		97.8	97.3	99.1	97.5	97.8
AY744471			96.8	97.2	97.2	97.2
AB190819				96.7	97.8	99.4
AB175809					96.9	97.2
AB038341						98.3

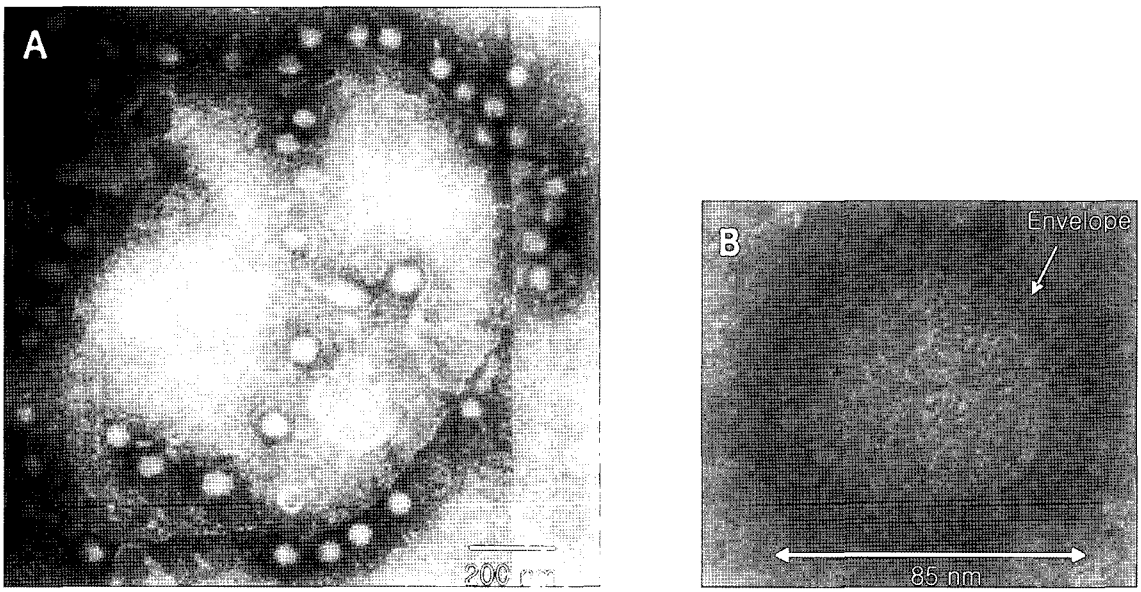

Fig. 3. Electron micrograph of TSWV from negatively stained leaf-dip preparations of *Nicotiana rustica* systemically infected with TSWV stained in 0.5% phosphotungustic acid. (A) Showing the presence of spherical virus particles; (B) one particle defined by a doubly membraned envelope.

Nucleotide sequences. Nucleotide sequences of TSWV-ch14 were submitted to the GenBank under the accession number of DQ453158. The most closely related strain was GenBank accession number AB010997 (Table 1).

Observation of particles by TEM. Topovirus-like particles around 85 nm in diameter in dip preparations from *N. rustica* were found (Fig. 3).

Discussion

In this study, a causal agent of TSWV-like symptoms on chrysanthemum plants was identified by determination of nucleotide sequences of partial genome of TSWV cloned from RNA extracted from infected plants, and the observation of particles by transmission electron microscope.

Typical TSWV-infected symptoms of leaf bronzing, foliar necrosis and stem necrosis were observed from 'Sinma' naturally infected with TSWV, which symptoms were accordance with previous reports (Sherman et al., 1997; Verhoeven et al., 1996). Meanwhile, in 'Hwarang', chlorotic spots, withering of axillary shoot and foliar necrosis were observed, but stem necrosis was not shown. Symptoms of leaf bronzing, vein necrosis along with top-shoot necrosis were observed from unknown pot-mum cultivar (data not shown), and chlorosis, necrosis or reddening of leaves were shown in 'Kasandra' (data not shown).

Common symptoms were chlorotic or necrotic leaf symptoms. Withering of 'Sinma' was shown to be caused by destruction of vascular system (Fig. 1H), thus 'Sinma' was the most damageable cultivar. On comparing the symptoms of cultivars by infection with TSWV, Sinma may be more susceptible than any other cultivars mentioned in the present study.

Nucleotide sequences of TSWV-ch14 was the most closely related with a TSWV-O strain (GenBank accession number AB010997) reported from Japan in 1994 (Tsuda et

al., 1994), inferring that TSWV may be responsible for the chrysanthemum cuttings introduced from Japan.

Verticillium known to causing wilting symptom in chrysanthemum (Hall and Busch, 1971) was not detected from chrysanthemums used for the determination of symptom by TSWV in the present study.

Nucleotide sequences and morphology of virus particles confirmed that chlorotic or necrotic leaf symptoms, wilting and stem necrosis occurring in chrysanthemum was caused by TSWV. This study also described various symptoms expressed from different chrysanthemum cultivars by infection with TSWV.

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