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Seed Transmission of *Chrysanthemum stunt viroid* in *Chrysanthemum*

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The presence of *Chrysanthemum stunt viroid* (CSVd) in seed and pollen of diseased chrysanthemum was demonstrated. In seeds infected male parent from crosses in May, CSVd was transmitted to 6.7% of the progeny seedlings, whereas if the female parent was infected, CSVd transmission rate was between 46.9 and 75.7%. A relatively high incidence of 94.4 to 96.0% seed transmission occurred when both parents were infected. In seeds infected male parent from crosses in December, no progeny seedlings were infected with CSVd, whereas if the female parent did, CSVd transmission rate was 1.5%. When both parents were infected, 6.9% seed transmission was occurred. The seed transmission rate depended on the temperature when the crosses were made. CSVd was not detected in the non-infected female parent pollinated with infected pollen but was transmitted to the progenies. This is the first report of seed-borne transmission of CSVd in chrysanthemum.

Keywords **:** chrysanthemum, *Chrysanthemum stunt viroid*, Seed transmission

Chrysanthemum is an important cut-flower world-wide. It is one of the three major cut-flower crops (Rose, Tulip, Chrysanthemum) (Facts and Figures, Bloemenveiling Aalsmeer, 2003). The sales of chrysanthemum ranks second in the Netherlands and third in Italy and Germany in sales (International Statistics Flowers and Plants, 2001). It accounts for about 31% of the total cut-flower cultivation area of 2,597 ha in Korea (Ministry of Agriculture and Forestry, 2005). The amount of export was totaled $8.6 million in 2005 (Ministry of Agriculture and Forestry, 2005).

Chrysanthemum stunt viroid (CSVd) is the most important viral disease in chrysanthemum worldwide. CSVd was firstly observed in South America in 1947, North America and Australia in 1951, the Netherlands in 1952, Belgium in 1972, Japan in 1977 and Brazil in 1990. Currently the pathogen occurs prevalently in the world (Lawson, 1987). CSVd has been present in Korea since 1997, and has spread nation wide. The infection rate of CSVd in 41 commercial chrysanthemum cultivars ranged from 9.8 to 66.4% in Korea. CSVd causes reduction of plant height, leaf size, flower size, and flower number by 32 to 50%, 26 to 35%, 14 to 36% and 14 to 75%, respectively (Chung et al., 2005).

CSVd as well as *Potato spindle tuber viroid* (PSTVd) and *Cucumber pale fruit viroid* (CPFVd) was found to be transmitted through seed and pollen of the tomato cultivars Rutgers and Najwcześniejszy (Fernow et al., 1970; Kryczyński et al., 1988). *Apple scar skin viroid* (ASSVd) or *Dapple apple viroid* (DAVd) was detected in the cotyledons and embryos of infected seeds (Hadidi et al., 1991). *Avocado sunblotch viroid* (ASVd) also has been transmitted through seeds (Wallace and Drake, 1962). On the other hand, transmission of CSVd through seeds of *Senecio cruentus*, *Chrysanthemum parthenium f. flosculosum* and *C. praealtum* in the laboratory (Brierley, 1953) was unsuccessful. Transmission of *Tomato planta macho viroid* (TPMVd) through tomato seeds (Galindo et al., 1982) and *Hop stunt viroid* (HSVd) through seeds of tomato and hop (Yaguchi and Takahashi, 1984) were also unsuccessful. CPFVd has been reported not to be transmitted through seed of cucumber (van Dorst and Peters, 1974). However, seed transmission of CSVd could not be demonstrated in *Chrysanthemum* species (Brierley, 1953). Therefore, in this study CSVd was assessed for seed transmission in chrysanthemum.

Materials and Methods

Cultivars used for crossing. Chrysanthemum cultivars Sharotte, Yeonja, and five breeding lines (6, 16, 27, 231501 and 241504) were used for making crosses. All cultivars and breeding lines used for making cross were germplasm preserved in Yesan Chrysanthemum Research Station, Yesan, Korea. Sharotte and Yeonja were bred from Japan. Lines 6, 16 and 27 were bred from crosses between cv. Grandpink and 231507 (cv. Hunt × cv. Delmonte). *Grandpink and Hunt were* bred from the Netherlands, and Delmonte was bred from Japan. Line 231501 was obred from crosses between cv. Vymini and line 223730. Line

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223730 was bred from open pollinated cv. Evrio. Vymini and Evrio were developed from the Netherlands. Line 241504 was obtained from crosses between the Netherlands cvs. Champion and Cosmosking.

The crosses were made in four combinations: healthy female×healthy male (H×H), healthy female×infected male (H×I), infected female×healthy male (I×H) and infected female×infected male (I×I). Five to ten pedicles per plant were used for the crosses. Independent crosses were made at two times: May and December. The temperature inside the greenhouse was approximately 25 (night) - 35°C (day) in May, and 25 (day) - 15°C (night) in December.

Pollination. Pollens were collected by painting brush on petridishes. Before flowers fully opened, one-third of the end of the flower leaf was cut off, and when the stigma opened by itself, pollen was rubbed onto the stigma using a brush. Seeds were harvested at 45 to 50 days after pollination.

Determination of transmission rate. The seed transmission rate of CSVd was determined by testing seedlings grown from seeds sown in potted sterilized soil (peat:perlite =1:1). To detect CSVd on both the seed coat and in germinated seedlings independently, seeds were placed on wet filter paper and then 4 days later the presence of CSVd was tested by reverse transcription polymerase chain reaction (RT-PCR).

Detection of CSVd from female chrysanthemum pollinated with pollen from plants infected with CSVd was conducted at 45 to 50 days after pollination.

RNA preparation and RT-PCR conditions. All CSVd detection conducted in this study was done using RT-PCR. For detection of CSVd by RT-PCR, RNA was prepared from 0.1 g of seedling leaves using a method described previously (Shiwaku et al., 1996). RNA was extracted using 1.2 ml of extraction buffer (0.1 M Tris-HCl, 50 mM EDTA, 2.8 M NaCl, pH 8.0) with 1% SDS, 0.004% of PVP and 600 μl of phenol:chloroform = 1:1 followed by purification by using CF11 cellulose in 30% ethanol in STE buffer (50 mM Tris-HCl, pH 7.2, 1 mM EDTA and 100 mM NaCl). RT-PCR was conducted as described previously (Chung et al., 2005).

The sequence of the reverse primer CSVd-R (5'-TTCT-TTCAAAGCAGCAGGGT-3') was complementary to nucleotides 36 to 55 of a CSVd isolate (GenBank accession no. AF394452) and that of the forward primer CSVd-F (5'-AAAGAAATGAGGCGAAGAAG-3') was homologous to nucleotides 56 to 75 of the CSVd isolate. For complementary DNA (cDNA) synthesis 1 μl of 10 pmol CSVd-R was added to 1 μg of RNA and then denatured at 70°C for 5 min. After denaturation 1 unit MuLV reverse transcriptase (Promega, USA), 10×transcription buffer and 2.5 mM of each dNTP were added on ice. The thermal conditions were as follows: denaturation at 94°C for 30 sec (2 min for the first cycle), annealing at 53°C for 30 sec and extension at 72°C for 1 min. The last cycle was extended for an additional 3 min at 72°C.

Sequence determination of CSVd. To verify the amplification of CSVd from infected seedlings, nucleotide sequences were determined using PCR products amplified from 2 infected seedlings from each of I×H, H×I and I×I crosses in Table 1. The amplified PCR products of the expected full length were eluted and cloned into the pGEM-T easy vector (Promega, 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). The nucleotide

Table 1. Percentage of *Chrysanthemum stunt viroid* infection in chrysanthemum seedlings grown from seeds harvested from chrysanthemum pollinated in May in the glasshouse[a]

Type of cross	Crosses Female × Male	No. of pedicels pollinated	No. of seedlings tested	No. of seedlings infected	% CSVd infection
I×I	Line 16 × Sharotte	24	75	72	96.0
	Line 6 × Sharotte	6	18	17	94.4
I×H	Line 6 × Line 231501	30	105	65	61.9
	Line 16 × Line 231501	30	103	78	75.7
	Line 27 × Line 231501	12	32	15	46.9
	Yeonja × Line 241504	12	33	22	66.7
H×I	Sharotte × Line 231501	12	30	2	6.7
H×H	Sharotte × Line 231501	4	10	0	0.0

[a] Seed were harvested at 45-50 days after pollination. CSVd was detected by reverse transcription polymerase chain reaction (Chung et al., 2005).

sequences were aligned using Clustal V using DNASTAR version 7.0.

Results

The efficiency of seed transmission of CSVd with a chrysanthemum cv. Sharotte, cv. Yeonja and 5 breeding lines crossed in May is shown in Table 1. A cross involving two infected breeding lines and infected Sharotte produced 94.4 to 96% infected seedlings while four crosses between infected breeding lines and healthy breeding lines produced 46.9 to 75.7% infected seedlings. Seeds that were pollinated with CSVd-infected chrysanthemum pollen that were produced 6.7% infected seedlings.

The seed transmission rate of CSVd crossed in December with cv. Yeonja is shown in Table 2. Crosses between infected male plants and healthy female plants produced 1.5% infected seedlings, while 6.9% seed transmission occurred when both parents were infected. 0% if the male was infected. No CSVd-infected seedlings were detected in seedlings derived from a cross between two healthy parents or from a cross between a healthy female and an infected male. The crosses in December showed a lower seed transmission rate compared to crosses performed in May.

In the seeds from a cross between two infected parents, CSVd was detected in 88.4% of the seed coats, while 72.5% of the progeny seedlings were infected with CSVd (Table 3). The percentage of pedicel containing ripened seeds ranged from 25.0 to 46.1% (Table 4). Crosses made using CSVd-infected parents produced a higher number of pedicels with ripened seeds than crosses involving healthy parents, but significantly more seeds per pedicel were produced from the H×H cross than from other combination (Table 4). CSVd was not detected in a healthy female parent pollinated with pollen from plants infected with CSVd (Fig. 1). CSVd was amplified from infected female parents (Fig. 1).

The nucleotide sequences of CSVd determined with PCR products amplified from infected seedlings were 100%

Table 2. Percentage of *Chrysanthemum stunt viroid* infection in chrysanthemum seedlings grown from seeds harvested from chrysanthemum cultivar Yeonj pollinated in December in the glasshouse[a]

Type of cross	No. of pedicels pollinated	No. of seedlings tested	No. of seedlings infected	% CSVd infection
I×I	39	58	4	6.9
I×H	37	68	1	1.5
H×I	40	31	0	0.0
H×H	26	10	0	0.0

[a]Seed were harvested at 45-50 days after pollination. CSVd was detected by reverse transcription polymerase chain reaction (Chung et al., 2005).

Table 3. Percentage of *Chrysanthemum stunt viroid* infection in seed coats or seedlings from seeds harvested from a cross between two infected parents at 45 days after pollination. Cross was conducted in May in the glasshouse

Type of cross	Crosses Female × Male	No. of seeds tested	No. of CSVd infected (%)	
			Seed coat	Seedling & seed coat
I×I	Line 16 × Line 231501	69	61 (88.4)	50 (72.5)

Table 4. Percentage of seed set per pedicel affected by *Chrysanthemum stunt viroid* infection of crossed parents in chrysanthemum cv. Yeonja pollinated in December in the glasshouse

Type of cross Female × Male	No. of pedicel pollinated	No. of pedicel with mature seed out of pollinated pedicel (%)	No. of total seeds ripened	Ave. no. ripened seeds per pedicel
I×I	39	18 (46.1)	62	3.4 c[a]
I×H	37	17 (45.9)	76	4.5 b
H×I	40	10 (25.0)	34	3.4 c
H×H	26	9 (34.6)	76	8.4 a

[a]Duncan's multiple range test, comparisons were made at a 0.05 alpha level. Means with the same letter are not significantly different.

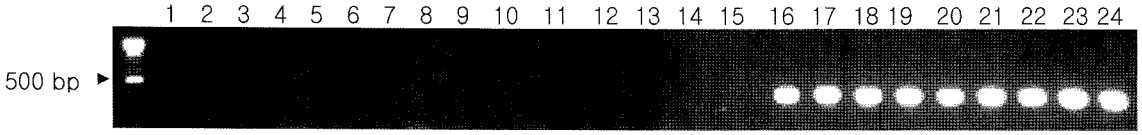

Fig. 1. Detection of *Chrysanthemum stunt viroid* in female parents at 45-50 days after pollination. Lanes 1 and 2, Healthy Sharotte (FM)×Healthy line 231501 (M); lane 3 to 15, Healthy Sharotte (FM)×CSVd-infected line 231501 (M); lane 16 to 20, CSVd-infected line 6 (FM)×Healthy line 231501 (M); lane 21 to 24, CSVd-infected line 16 (FM)×CSVd-infected Sharotte (M). RT-PCR products were examined by 1.5% agarose gel electrophoresis. The size marker is shown on the left.

identical to that of the CSVd Korean isolate (GenBank accession no. AF 394452).

Discussion

Bennett (1969) reviewed evidence that in many instances male and female gametophytes as well as developing embryos escape virus infections because of their weak symplastic connections with the sporophytes. Meanwhile viroid RNA is extremely small and is likely to be translocated through these weak connections. Kryczyński et al. (1983) suggested that viroids are seed transmitted pathogens because of their affinity to meristematic cells which probably results from the extremely small size of the viroids.

In the present study we have shown that CSVd is transmitted to progeny of crosses which include an infected parent. In crosses done in May, where the male parent was infected, CSVd was transmitted to about 6.7% of the progeny seedlings, and a relatively high incidence (96.0 to 94.4%) of seed transmission occurred when both parents were infected. On the other hand, crossing of PSTVd-infected tomato plants showed that the highest incidence (11%) of PSTVd was observed in seedlings in which both parents were infected (Singh, 1970). Meanwhile when the male parent was infected the incidence of infected progeny (9%) was higher than when the female parent was infected (6%) (Singh, 1970). In our study, CSVd showed the opposite results to PSTVd in the transmission rate when either parent was infected. Similarly when both parents were infected, the highest incidence of seed transmission occurred in both CSVd and PSTVd.

In a previous report (Fernow et al., 1970) the seed transmission of PTSVd was not correlated with variety of potato, even though the percentage transmission was variable in the different collections from 0 to 100%. But we can not rule out the possibility that chrysanthemum cultivars differ in their ability of transmitting CSVd through seeds, because in the I × H type cross, efficiency of seed transmission ranged from 46.9 to 75.7% for different crosses involving different breeding lines for female parents (Table 1). But, the speculation is difficult to discuss these results since no various cultivars or no enough amounts of seedlings were used for this study.

The progeny from the crosses made in December showed relatively lower seed transmission rate of CSVd than those done in May, suggesting that temperature under which crosses were made affected the seed transmission rate of CSVd. Cv. Yeonja, which was used for both May and December, supports the conclusion. It is known that replication of CSVd and *Chrysanthemum chlorotic mottle viroid* is reduced at 5°C (Paludan, 1985). CSVd-infected chrysanthemum grown under 10 to 15°C for 2 months showed lower concentration of CSVd than when grown under 25 to 35°C (Chung et al., 2006). The low seed transmission rates of CSVd from seedlings obtained from crosses done in December can be resulted from the reduced replication of CSVd caused by low temperature under which the crossed parents were grown.

The low percent infection of chrysanthemum seedlings through pollination may be explained by CSVd-infected pollen being less viable, so that less of it can reach the embryo. This suggestion is supported by the significantly fewer number of seed set per pedicel from the H×I or I×I combinations than from the H×H or I×H combinations (Table 4). The average number of seeds per pedicel decreased from CSVd-infected maternal plants, but the number of pedicels which contained ripened seeds from maternal plants of I×I or I×H was higher than that from H×I or H × H crosses. This might be due to the poor growth of CSVd-infected chrysanthemum, which accelerated early ripening of stigma, resulting in an increased chance of seed set in the pedicel.

Seed infection occurring through pollen while the female parent remains virus-free has been reported for several virus-infected host plants (Bennett, 1969; Johansen et al., 1994; Sdoodee and Teakel, 1988). Similarly CSVd was detected from pollen grains by RT-PCR (data not shown). CSVd-infected pollen could not infect maternal tissue by pollination even though CSVd was transmitted to the progeny.

Chrysanthemum cultivars have been developed by artificial crosses between cultivars. Our findings emphasize the need for using CSVd free plants as parents in chrysanthemum breeding program.

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