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Chrysanthemum stunt viroid Induces the Accumulation of Small RNAs Associated with RNA Silencing in Infected Chrysanthemum

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***Chrysanthemum stunt viroid* (CSVd) induced systemic symptoms on chrysanthemum. We detected small RNAs of approximately 22 nucleotides with sequence specificity to CSVd in chrysanthemum infected with CSVd: an indication of the presence of RNA silencing. Regardless of symptom differences associated with CSVd, the small RNAs distributed similarly in amount. Small RNAs were detected with partial-length or full-length probes, indicating that they are not restricted to specific viroid regions but likely representing most of the viroid molecule.**

Keywords **:** *Chrysanthemum stunt viroid*, small RNAs

Viroids are small single-stranded, covalently closed circular RNA molecules of 246 to 375 nt which is not encapsidated in a protein coat (Diener, 1993). Viroid species are clustered into the families *Pospiviroidae* and *Avsunviroidae* (Mayo, 1999), for which viroids replicate in nucleus and chloroplast, respectively (Flores et al., 2005). Viroids replicate in three steps through an RNA-based rolling-circle mechanism (Branch and Robertson, 1984; Bussière, 1999): synthesis of longer-than-unit strands catalyzed by host RNA polymerase; processing to unit-length; and circularization. Based on these findings, double stranded RNAs (dsRNAs) are transiently formed at several stages of *Chrysanthemum stunt viroid* (CSVd) replication and they could be the target of an RNase III-like nuclease (Branch and Robertson, 1984).

RNA silencing can be subclassified into RNA-mediated transcriptional gene silencing (TGS) and post transcriptional gene silencing (PTGS) (Baulcombe, 2004). TGS occurs when dsRNA with sequence homology to a promoter is produced, leading to de novo DNA methylation of the promoter region of the structural gene (Mette et al., 2000). On the other hand, PTGS yields reduced steady state levels of targeted host or viral cytoplasmic RNA (Lindbo et al., 1993), and may be mediated through a host-encoded RNA-dependent RNA polymerase (RdRp) and RNA helicase (Dalmay et al., 2000, 2001). A plant virus can be both the inducer and target of RNA silencing (Baulcombe, 1996). It has been reported in plants and other organisms such as fungi and animals (Vance and Vaucheret, 2001). In plants it serves as an antiviral defense (Baulcombe, 1999; Ratcliff et al., 1999). Evidence of PTGS has focused on the production of characteristic small interfering RNAs (siRNAs) of 21-26 nucleotides by the action of a ribonuclease Dicer on dsRNA (Hamilton and Baulcombe, 1999).

Initial evidence of PTGS targeting PSTVd, included in family *Pospiviroid*, in infected tomato was reported with detection of PSTVd specific siRNAs (Itaya et al., 2001; Papaefthimiou et al., 2001). Small RNAs have been detected in plants infected by three members of the family *Avsunviroidae*, *Peach latent mosaic viroid* (PLMVd), *Chrysanthemum chlorotic mottle viroid* (CChMVd) (Martínez et al., 2002) and *Avocado sunblotch viroid* (ASBVd) (Markarian et al., 2004).

In this study, we have examined whether the *Chrysanthemum stunt viroid* (CSVd) is able to trigger a PTGS in their natural host chrysanthemum by examining the production of small RNAs.

Materials and Methods

Plant materials used for siRNA analysis of different symptoms. Chrysanthemum cultivars 'Sharotte' and 'Sinma' were collected from commercial chrysanthemum fields in Goyang, Kyeonggi Province, and were maintained in a greenhouse. Single infection with CSVd was confirmed by RT-PCR using a method described previously (Chung et al., 2005). To investigate the accumulation level of small RNAs according to symptom differences associated with CSVd in different cultivars, vein yellowing 'Sharotte', leaf curling 'Sharotte' and symptomless 'Sinma' were used for RNA preparations. Healthy chrysanthemum was used as a control.

Inoculation of chrysanthemum with RNA transcripts. To examine the amount of small RNAs as a relation to the concentration of CSVd, we inoculated virus- and viroid-free chrysanthemum cultivar Sinma with RNA transcripts of CSVd prepared in vitro. In vitro RNA transcripts were

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prepared with a full length CSVd clone (GenBank accession no. AF394452) using mMESSAGE mMACHINE T7 (Ambion, USA) according to the manufacture's instructions. Stems were slashed using a blade and one hundred nanograms of RNA was injected to each plant. Those plants were grown in a glass-house set at 25°C. Samples from systemic leaves were collected at 17 days, 24 days, 31 days and 38 days post inoculation (dpi).

Preparation of RNA samples, probes and Northern blot analysis. Small RNAs were prepared as described in previous reports (Canto et al., 2002; Hamilton and Baulcombe, 1999). About 0.5 g of leaf tissue was ground in liquid nitrogen with mortar and pestle, and was suspended in 1.5 ml of extraction buffer (0.1 M LiCl, 0.1 M Tris HCl, pH 8.0, 0.01 M EDTA and 1% SDS), prewarmed at 80°C before using. After three times of phenol and chloroform treatment, the supernatant was mixed with a same volume of 10% PEG 8000 in 1 M NaCl solution and then put it on ice for 20 min. Nucleic acids was precipitated by adding a same volume of isopropanol. The pellet was collected by centrifugation at 13,000 rpm for 20 min followed by washing with 70% ethanol.

Small RNA preparations were separated by electrophoresis through 15% polyacrylamide 7 M urea 0.5X Tris-borate EDTA gels, blotted to Hybond N+ membranes (Amersham, UK), and fixed by ultraviolet cross-linking. The blotted membrane was pre-hybridized in ULTRAhyb (Ambion, USA) at 45°C for 30 min, and hybridization was made in the same solution with single stranded RNA probes at 42°C overnight.

Antisense RNA probe was prepared from a CSVd clone, after linearization with *Sal* I, using T7 RNA polymerase. Sense RNA probe was also obtained from the same clone, after linearization with *Nco*I, using SP6 RNA polymerase. A partial length of probe was prepared with an antisense RNA ranging from nucleotides 62 to 283 (222 bp) of a CSVd isolate (GenBank accession no. AF394452). Synthesis of the probes and processing of the blot with digoxigenin-labeled probes were performed according to the manufacture's instructions (Roche, Basel, Switzerland). Probes were denatured by boiling for 5 min.

Results

To test whether CSVd could trigger PTGS in its natural host, CSVd-infected chrysanthemum was analyzed for the presence of small RNAs. Figure 1 shows RNA extracts from CSVd-infected tissue hybridized with RNA probes specific for detecting positive or negative CSVd strands. Small RNAs were detected in diseased plants by both polarity of full-length CSVd probes (Fig. 1A and B) and partial-length of antisense probe (Fig. 1C); however, they were not in the healthy control plants (Fig. 1). The accumulation levels of the small RNAs were similar, regardless of symptom levels induced in different cultivars (Fig. 1).

We examined the presence of small RNAs in chrysanthemum plants as time passes after inoculation of RNA transcripts. Twenty-two nucleotide RNAs of CSVd was detected 24 dpi of 'Subangryuk', and the amount was higher at 31 dpi than at 24 dpi (3 or 10-fold higher for two

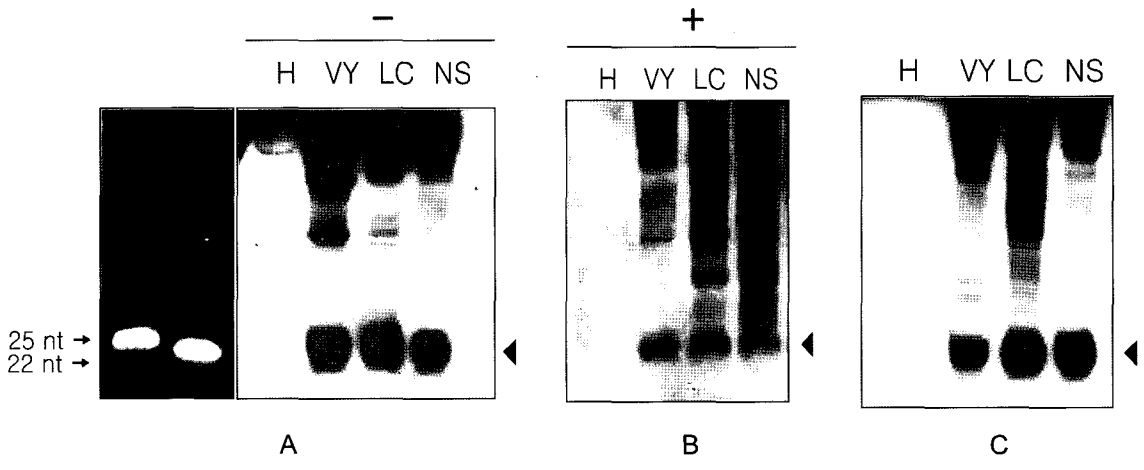

Fig. 1. Northern analysis for detection of PTGS-typical small RNAs from *Chrysanthemum stunt viroid* infected chrysanthemum. RNA preparations from healthy (H) and CSVd-infected chrysanthemum plants from 'Sharotte' revealing vein yellowing (VY) or leaf curling (LC), and 'Sinma' infected without symptom (NS) were separated in a 15% polyacrylamide-7 M urea gel. (A) The hybridization probe was the full-length CSVd minus-strand (−) RNA probe labeled with digoxigenin. Positions of the 22 nt and 25 nt DNA markers are at the left. (B) The hybridization probe was the full-length CSVd plus-strand (+) RNA probe (C) The hybridization probe was the partial-length (222bp) of CSVd minus-strand (−) RNA probe labeled with digoxigenin. The signals corresponding to the small RNAs were indicated by arrowheads at the right.

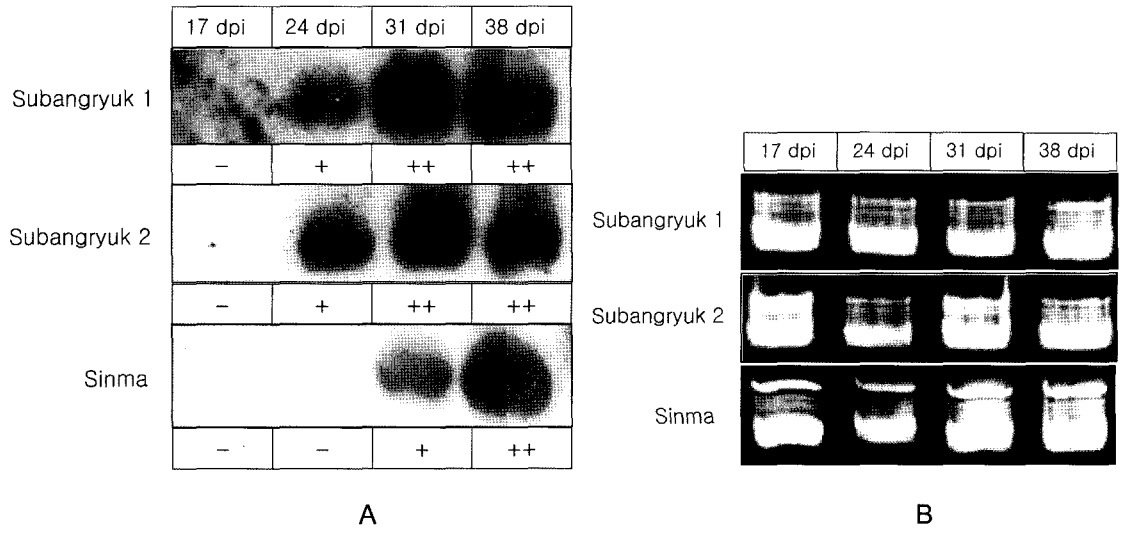

Fig. 2. Northern analysis for detection of PTGS-typical small RNAs from chrysanthemum inoculated with CSVd RNA transcripts as time passes. Chrysanthemum cvs. 'Subangryuk' and 'Sinma' were inoculated with in vitro RNA transcripts of *Chrysanthemum stunt viroid*, leaves were harvested at 17, 24, 31 and 38 days post-inoculation (dpi) to prepare RNA. Subangryuk 1 and 2 mean two independent experiments. (A) Samples were hybridized with a full-length CSVd (−) RNA probe labeled with digoxigenin. (B) Ethidium bromide stained gel shows loading of sample preparations.

independent experiments, respectively) (Fig. 2). In 'Sinma', we found that samples taken at day 38 contained 5-fold more small RNAs than those taken at day 31 (Fig. 2). The amount of small RNAs increased with time for another 7 days following the first detection in both cultivars (Fig. 2).

Discussion

The present study showed the presence of small CSVd homologous RNAs of both polarities of CSVd genome in infected chrysanthemum. This indicats that CSVd induced PTGS in chrysanthemum like other viroids such as PSTVd, ASVd or CChMVd (Itaya et al., 2001; Markarian et al., 2004; Martínez et al., 2002).

We detected small RNAs of approximately 22 nt with sequence specificity to CSVd in infected chrysanthemum but not in healthy control plants (Fig. 1). Its length was in the range of characteristic small interfering RNAs (siRNAs) of 21-26 nt (Hamilton and Baulcombe, 1999): Length of small RNAs triggered by PSTVd (Papaefthimiou et al., 2001); CChMVd (Martínez et al., 2002) and ASBVd (Markarian et al., 2004) were 22-23 nt, 21-23 nt and 22 nt, respectively. In the light of the consistent production of small RNAs in all other RNA silencing systems (Hamilton and Baulcombe, 1999; Papaefthimiou et al., 2001), we could conclude the presence of CSVd-specific small RNAs in the infected chrysanthemum plants as an indication of viroid-induced RNA silencing.

The small RNAs could be detected with partial or full-length CSVd-specific probes (Fig. 1), showing that they form a population of sequences not restricted to specific viroid regions but likely representing most of the whole viroid molecule. Small RNAs were also detected with probes of both polarity (Fig. 1), indicating that negative and positive strands were represented in these RNAs.

In this study 22 nt CSVd RNA was accumulated to a similar extent regardless of symptom differences (Fig 1). No correlation between the virulence of the viroid strain and the *in vivo* concentration of the small RNAs was reported for PSTVd (Papaefthimiou et al., 2001). Above results suggested that accumulation of small RNAs was not directly involved in symptom development. On the other hand, in avocado, tissues with the different symptom expressions, characterized by the presence of different predominant ASBVd variants, induced small RNAs at different levels (Markarian et al., 2004). Further investigations are required to define the relationship between small RNAs and virulence of viroid.

PTGS was induced at the early stage of CSVd replication. The first occurrence of small RNAs was dependent on cultivars (Fig. 2). Twenty-two nucleotide RNAs of CSVd was detected 24 dpi of 'Subangryuk', and continued to accumulated for another 7 days in systemically infected leaf: On the other hand, in 'Sinma', small RNAs was detected at 31 dpi and also increased for another 7 days. Those results suggested that late induction of RNA silencing in 'Sinma' compared with 'Subangryuk' might indicate that 'Sinma' is much more susceptible to CSVd than 'Subangryuk' is, because PTGS serves as an antiviral defense (Baulcombe, 1999).

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