***In vitro* Evaluation of Small RNA Accumulation in Chrysanthemum Mediated by Chrysanthemum Stunt Viroid**

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A comparative analysis of stunting was conducted at various temperatures to develop a simple and reliable method for evaluating the disease symptoms of the chrysanthemum stunt viroid (CSVd). CSVd-free plants had significantly greater plant height than CSVd-carrying plants at 22°C and 24°C. No significant differences in plant height were detected at high and low temperatures. These results suggest that the *in vitro* evaluation of plant growth at an appropriate temperature might provide a useful method for CSVd diagnosis. In addition, the intensity of the small RNA hybridization signal obtained by northern blot analysis using a full-length CSVd RNA as a probe indicated synchronous behavior that correlated with the degree of growth suppression. These results suggest that a small RNA from CSVd may lead directly to the suppression of plant growth, with a similar function to siRNAs in chrysanthemum.

Keywords : chrysanthemum, CSVd, *in vitro*, northern hybridization, small RNA

INTRODUCTION

The chrysanthemum, *Chrysanthemum morifolium* Ramat., is one of the most important cut flowers alongside the rose and carnation. Most commercial cultivars are cultivated at a high density all year round in a protected facility such as a plastic film greenhouse, pipe frame greenhouse, or glass greenhouse. Protected cultivation facilitates the control of disease and intensive propagation. However, the spread of diseases caused by viroids cannot be controlled in these facilities, because resistance genes or suitable agrochemicals are not available. Stunting disease is caused by the chrysanthemum stunt viroid (CSVd), and is one of the most serious diseases that affects cultivated chrysanthemums (Matsushita et al., 2007). The symptoms of this disease have been reported as including stunted plant height and other morphological or physiological changes such as reduced flower size, flower color bleaching, and a perturbed photoperiodic response (Horst et al., 1977; Hosokawa et al., 2004b). CSVd has a single-stranded circular RNA genome containing 348-356 nucleotides without any coding regions (Matsushita et al., 2007). Previous studies of CSVd have focused on the variation in its nucleotide sequence and methods for its detection and elimination from host plants (Hosokawa et al., 2004a, 2006; Matsushita et al., 2007; Nakashima et al., 2007). However, few reports have evaluated quantitative changes in the growth of CSVd-infected plants because of the difficulty of preparing uniform environmental conditions during the cultivation and growth stages. Thus, it is necessary to develop an appropriate cultivation system to understand the

importance of CSVd RNA for host plants.

The mechanisms whereby CSVd causes symptoms remain unclear, because many reports described that symptom severity was not correlated with accumulation levels of viroid RNAs (Gruner et al., 1995; Góra et al., 1996; Rodio et al., 2006). RNA silencing induced by viroid-specific small RNAs is considered to be one of the most important factors that cause viroid symptoms. A large volume of short interfering RNAs (siRNAs) that correspond to specific regions of the viroid genome have been found to accumulate in host plants in recent studies of plants infected with several species of viroids (Itaya et al., 2007; Machida et al., 2007, 2008; Martin et al., 2007). These siRNAs may induce posttranscriptional gene silencing (PTGS) in the host plant to produce symptoms. However, there have been no reports of a link between the quantity of siRNA in CSVd-infected chrysanthemum and the degree of disease symptoms because of the difficulties mentioned above.

In vitro culture systems have many advantages for evaluating plant growth because they can generate highly controlled uniform conditions in terms of the temperature, photoperiod, and nutrient supply. There have been some reports of the *in vitro* estimation of plant disease symptoms. For example, Takatsu et al. (2000) successfully evaluated the susceptibility to white rust of 12 chrysanthemum cultivars using an *in vitro* culture system.

The objective of the current study was to establish a simple and reliable method for evaluating chrysanthemums exhibiting stunting as a symptom of CSVd disease using an *in vitro* culture system. We also aimed to evaluate the comparative effect of CSVd-derived small RNA accumulation on the stunting of host plants.

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MATERIALS AND METHODS

Plant material

Chrysanthemum morifolium (Ramat.) cv. 'Mistletoe' was used as the plant material. 'Mistletoe' is an indicator cultivar for CSVd, which exhibits several symptoms including stunted plant height. CSVd-free plants were maintained aseptically *in vitro* with 1/2 Murashige and Skoog (MS) (Murashige and Skoog, 1962) solid medium (MS macronutrients at half strength) that contained 0.3% (w/v) gelrite and 3% (w/v) sucrose (pH 5.8) at 25°C, using a 16 h photoperiod illuminated by a cool white fluorescent light (3,500 lx). Young shoots were cut from a mother plant and grafted onto a CSVd-carrying 'Hakuju' chrysanthemum plant to achieve infection. After confirmation of CSVd RNA in the scion by direct RT-PCR (Hosokawa et al., 2006), a shoot was cut from the grafted mother plant and propagated vegetatively *in vitro* for six months.

Culture conditions

During preculture, young 1 cm shoots with two expanded leaves were excised from cuttings that were propagated from CSVd-free and CSVd-carrying plants, which were then placed into 50 mL of 1/2 MS solid medium in a plastic box (6×6×10 cm) at 25°C with a 16 h photoperiod (3,500 lx) to induce root formation after one week. After confirmation of root formation, these boxes were transferred to the growth chamber (LH-100-RD; NK System, Osaka, Japan) and cultured at 5, 15, 18, 20, 22, 24, 26, or 28°C under continuous cool white fluorescent light (4,000 lx). After one month of culture, the plant height was measured as a CSVd disease symptom. The third leaf from the top of plant was then excised, frozen in liquid nitrogen immediately, and maintained in a deep freezer at -80°C prior to RNA extraction. Five plants were cultured in each temperature, and experiments were conducted for three times.

In vitro transcription of DIG-labeled CSVd RNA

Total RNA was extracted from CSVd-carrying plant leaves using a Qiagen RNeasy Plant Mini Kit (Qiagen, CA, USA). The cDNA of the whole CSVd sequence was amplified by RT-PCR using the primers CSVd-ZF (5'-3': TGTGGTGCACCTCCTGACCCT) and CSVd-ZR (5'-3': GGAACCACAAGTAAGTCCCG) according to the method of Matsushita et al. (2007). The cDNA was then used as a template for PCR amplification of the whole CSVd sequence by adding a T7 promoter to the CSVd-ZF priming site using the primers T7/CSVd-ZF (5'-3': TAATACGACTCACTATAGGGTGTGGTGCACCTCCTGACCCT) and CSVd-ZR. DIG-labeled (—) CSVd linear RNA was transcribed with T7 RNA polymerase using a DIG Northern Starter Kit (Roche Diagnostics, Basel, Switzerland), according to the manufacture's instructions, before being used as an RNA probe.

Northern blot hybridization

Low molecular weight RNAs were extracted from the leaves of cultured plants using a PureLink™ miRNA Extraction Kit (Invitrogen, CA, USA). Northern blot analysis was conducted to detect CSVd-derived siRNA. Two micrograms of low molecular RNAs were separated

on a 12% denatured polyacrylamide gel containing urea (12×12 cm). The separated RNAs were electrotransferred onto a Hybond-N⁺ nylon membrane (GE Healthcare, Buckinghamshire, UK) and hybridized overnight with the DIG-labeled (—) CSVd linear RNA at 37°C. Washing and detection of the chemiluminescent signal was performed using a DIG Northern Starter Kit, according to the manufacture's procedure.

RESULTS AND DISCUSSION

The cut shoots produced various levels of growth depending on the culture temperature. The data obtained in each temperature was analyzed collectively, because the significant differences between experiments were not observed. The temperature severely affected the growth of CSVd-free plants (Fig. 1). The plants grew vigorously and extended to a maximum of 10.1 cm at 22°C, whereas the slowest level of growth was 4.2 cm at 5°C. In contrast, CSVd-carrying plants grew slowly at all temperatures to a height of 3.3–5.7 cm. A comparative analysis of plant height showed that CSVd-free plants grew significantly higher than CSVd-carrying plants at 22°C and 24°C ($P < 0.01$). These results suggest that CSVd made host plants insensitive to the culture temperature, leading to growth suppression as a disease symptom. Other symptoms, such as leaf yellowing and necrotic blotching, were observed infrequently at all temperatures. Handley and Horst (1988) reported that frequent severe necrotic symptoms affected the leaves at high temperatures between 29 and 32°C in 'Bonnie Jean'. The differences in the expression of symptoms might be attributable to the different growing conditions (*in vitro* or not) and the cultivar used. As mentioned above, the *in vitro* evaluation of plant growth is considered to be a useful method for CSVd diagnosis.

In order to clarify the correlation of CSVd RNA and disease symptoms, RNA analyses were conducted. In the preliminary experiment, quantitative real-time RT-PCR to detect genomic CSVd RNA was performed for all the individuals. However, too large quantitative variations of CSVd in individuals at each temperature prevent comparison each other (data not shown). In potato tuber spindle

Fig. 1 Plant height of CSVd-free and CSVd-carrying plants at eight temperatures after one month of culture. □: CSVd-free plants, ■: CSVd-carrying plants. Vertical bars represent the S.E. *, ** indicate significant differences within treatment at the 5% and 1% levels using the Student's *t*-test.

viroid (PSTVd) infected tomato, symptom severity did not correlate with viroid accumulation (Góra et al., 1996). Gómez et al. (2008) exhibited that the disease intensity of hop stunt viroid (HSVd) was independent of HSVd accumulation level in *Nicotiana benthamiana*. These observations were consistent with our result. Therefore, comparative analysis of small RNA content was conducted. The yield of small RNA increased synchronously with the degree of growth suppression. Consequently, total two to 8 leaves, from one leaf per plant, were used for 2 µg of small RNA extraction. The northern blot analysis detected the hybridization signals of small RNAs in CSVd-carrying plants at all temperatures, whereas they were absent in CSVd-free plants (Fig. 2). Chung et al. (2006) detected small RNAs with sequences specific to CSVd in chrysanthemums infected with CSVd. This report agreed with our results. Hybridized small RNAs with 20–25 nucleotides were identical to the partial sequence of a full-length CSVd-specific probe and they were considered to be CSVd-induced siRNAs. We conducted quantitative analysis using the image analysis program ImageJ Ver. 1.45 to compare the hybridization densities at eight temperature conditions (<http://rebweb.nih.gov/ij>). The intensity of the hybridization signal was severely affected by the growing temperature (Fig. 3). The signal intensity increased up to 22°C, before decreasing to 28°C. These results suggest that small RNA accumulation was linked directly with the suppression of plant growth. Recent studies have suggested that small viroid RNA can act like siRNAs and mediate the silencing of host genes to cause disease symptoms (Flores

Fig. 2 Northern blot analysis of small RNAs hybridized with a full-length CSVd-specific probe at eight temperatures.

Fig. 3 Comparative intensity of the hybridization signal obtained by northern blot analysis at eight temperatures. The signal intensity is shown relative to the value at 15°C.

et al., 2004; Markarian et al., 2004). CSVd-derived small RNAs might have a similar function to siRNAs in suppressing growth. Navarro et al. (2009) identified two distinct siRNAs in pathogenic RNA-infected plants, i.e., primary siRNAs that resulted from the cleavage of an initial trigger RNA by Dicer-like enzymes (DCLs) and secondary siRNAs, the biogenesis of which required an RNA-dependent RNA polymerase (RDR). Indeed, the scion of transgenic *N. benthamiana*, in which the RDR is constitutively silenced, grew well on the stock *N. benthamiana* carrying HSVd (Gómez et al., 2008). Both enzymes were essential for pathogenesis in a plant cell system and their bioactivities were considered to be dependent on plant vigor. Therefore, it is likely that a correlation between the signal intensity and the degree of growth suppression would occur at different temperatures.

In this experiment, we established a simple method for the phenotypic evaluation of CSVd disease symptoms using an *in vitro* culture system. We used this culture system to demonstrate that the accumulation of CSVd-derived small RNAs was correlated with growth suppression as a disease symptom. We are now identifying the sequences of these small RNAs. Further experiments are now being conducted to clarify the consensus sequences in CSVd-derived small RNAs and host plant-derived mRNAs.

REFERENCES

- Chung, B. N., Choi, G. S., Kim, K. T. 2006. *Chrysanthemum stunt viroid* induces the accumulation of small RNAs associated with RNA silencing in infected chrysanthemum. *Plant Pathol. J.* **22**: 235–238.
- Flores, R., Delgade, S., Gas, M. E., Carbonell, A., Molina, D., Gago, S., De La Pena, M. 2004. Viroids: the minimal non-coding RNAs with autonomous replication. *FEBS Lett.* **567**: 42–48.
- Gómez, G., Mañe, G., Pallás, V. 2008. Viroid-induced symptoms in *Nicotiana benthamiana* plants are dependent on RDR6 activity. *Plant Physiol.* **148**: 414–423.
- Góra, A., Candresse, T., Zagórski, W. 1996. Use of intramolecular chimeras to map molecular determinants of symptom severity of potato spindle tuber viroid (PSTVd). *Arch. Virol.* **141**: 2045–2055.
- Gruner, R., Fels, A., Qu, F., Zimmat, R., Steger, G., Riesner, D. 1995. Interdependence of pathogenicity and replicability with potato spindle tuber viroid. *Virology* **209**: 60–69.
- Handley, M. K., Horst, R. K. 1988. The effect of temperature and light on chrysanthemum stunt viroid infection of florists chrysanthemum. *Acta Hort.* **234**: 89–97.
- Horst, R. K., Langhans, R. W., Smith, S. H. 1977. Effects of chrysanthemum stunt, chlorotic mottle, aspermy and mosaic on flowering and rotting of chrysanthemums. *Phytopathology* **67**: 9–14.
- Hosokawa, M., Otake, A., Oishi, K., Ueda, E., Hayashi, T., Yazawa, S. 2004a. Elimination of chrysanthemum stunt viroid from an infected chrysanthemum cultivar by shoot regeneration from a leaf primordium-free shoot apical meristem dome attached to a root tip. *Plant Cell Rep.* **22**: 859–863.
- Hosokawa, M., Ueda, E., Ohishi, K., Otake, A., Yazawa, S. 2004b. Chrysanthemum stunt viroid disturbs the photo-periodic response for flowering of chrysanthemum plants. *Planta* **220**: 64–70.
- Hosokawa, M., Matsushita, Y., Uchida, H., Yazawa, S. 2006.

- Direct RT-PCR method for detecting two chrysanthemum viroids using minimal amounts of plant tissue. *J. Virol. Meth.* **131**: 28–33.
- Itaya, A., Zhong, X., Bundschuh, R., Qi, Y., Wang, Y., Takeda, R., Harris, A. R., Molina, C., Nelson, R. S., Ding, B. 2007. A structured viroid RNA serves as a substrate for dicer-like cleavage to produce biologically active small RNAs but is resistant to RNA-induced silencing complex-mediated degradation. *J. Virol.* **81**: 2980–2994.
- Machida, S., Yamahata, N., Watanuki, H., Owens, R. A., Sano, T. 2007. Successive accumulation of two size classes of viroid-specific small RNA in potato spindle tuber viroid-infected tomato plants. *J. Gen. Virol.* **88**: 3452–3457.
- Machida, S., Shibuya, S., Sano, T. 2008. Enrichment of viroid small RNAs by hybridization selection using biotinylated RNA transcripts to analyze viroid induced RNA silencing. *J. Gen. Plant Pathol.* **74**: 203–207.
- Markarian, N., Li, H. W., Ding, S. W., Semancik, J. S. 2004. RNA silencing as related to viroid induced symptom expression. *Arch. Virol.* **149**: 397–406.
- Martin, R., Arenas, C., Daros, J. A., Covarrubias, A., Reyes, J. L., Chua, N. H. 2007. Characterization of small RNAs derived from *Citrus exocortis viroid* (CEVd) in infected tomato plants. *Virology* **367**: 135–146.
- Matsushita, Y., Tsukiboshi, T., Ito, Y., Chikuo, Y. 2007. Nucleotide sequences and distribution of *Chrysanthemum stunt viroid* in Japan. *J. Jpn. Soc. Hort. Sci.* **76**: 333–337.
- Murashige, T., Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**: 473–497.
- Nakashima, A., Hosokawa, M., Maeda, S., Yazawa, S. 2007. Natural infection of *Chrysanthemum stunt viroid* in dahlia plants. *J. Gen. Plant Pathol.* **73**: 225–227.
- Navarro, B., Pantaleo, V., Gisela, A., Mozon, S., Dalmay, T., Gyorgy, B., Serio, F., Burgyn, J. 2009. Deep sequencing of viroid-derived small RNAs from grapevine provides new insights on the role of RNA silencing in plant-viroid interaction. *PLoS ONE* **4**: 1–12.
- Rodio, M. E., Delgado, S., Flores, R., Di Serio, F. 2006. Variants of *Peach latent mosaic viroid* inducing peach calico: Uneven distribution in infected plants and requirements of the insertion containing the pathogenicity determinant. *J. Gen. Virol.* **87**: 231–240.
- Takatsu, Y., Ohishi, K., Tomita, Y., Hayashi, M., Nakajima, M., Akutsu, K. 2000. Use of chrysanthemum plantlets grown *in vitro* to test cultivar susceptibility to white rust, *Puccinia horiana* P. Hennings. *Plant Breed.* **119**: 528–530.