Improving the *Chrysanthemum stunt viroid* (CSVd) resistance of chrysanthemum

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**Summary**: Viroids, the smallest RNA pathogen, infect different kinds of horticultural and agricultural crop, and lead to marked quality deterioration in the case of symptomatic infection. It is apparent that their autonomous replication and systemic movement in plants fully depend on the host machinery, since the pathogens lack their own protein-coding genes, whereas how viroids cause disease remains elusive. A model of short interfering RNA (siRNA) derived from viroid RNA that disrupts host gene expression, followed by an abnormal morphological appearance, is more acceptable than those previously proposed. On the other hand, the application of siRNA to block viroid replication is a plausible strategy for the molecular breeding of viroid-resistant plants. Recent advancements in the knowledge of viroid biology, as well as our approach to molecular breeding for *Chrysanthemum stunt viroid* (CSVd)-resistant chrysanthemum, are briefly reported.

**Keywords**: Viroid, siRNA, Molecular breeding, *Chrysanthemum*

**Introduction**

Viroids are plant-pathogenic, single-stranded, covalently closed, circular RNA molecules that range in size from 250 to 400 nucleotides, without any apparent open reading frame coding for proteins (Di Serio et al., 2014). Because of their simple genomic composition, it is believed that replications and movements of viroids in plants definitely depend on the host machinery (Flores et al., 2008). More than thirty species belonging to two families, *Pospivioidae* and *Avsunviroidae*, have been identified, and most of them are members of *Pospivioidae* (NCBI taxonomy browser for viroid: http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?p=7&id=12884). In spite of their naked RNA molecule without a protein coat, viroids generally show physical robustness, e.g., the infectious capacity of *Tomato chlorotic dwarf viroid* (TCDVd) was eventually lost after boiling infected plant extracts for more than 40 min (Matsushita, 2011). They can enter plants through mechanical injury when contaminated pruning scissors or other tools are used. Thus, careful field management of disease and establishing viroid-resistant varieties are required. CSVd, a member of *Pospivioidae*, causing the stunted growth of many varieties of chrysanthemum (*Dendranthema x morifolium* Ramat.), was initially identified in 1945 in the USA and is now distributed globally (Matsushita, 2011). Since chrysanthemum is ranked as the second most economically important cut flower in the world, much effort is needed to establish resistant cultivars. In the present article, we briefly summarize the current biological knowledge of viroids and introduce our research approach for the molecular breeding of CSVd-resistant chrysanthemum.

**Symptoms and proposed mechanisms of viroid-host interactions**

Symptoms of plants infected with viroids are macroscopically similar to those of plants infected with many plant viruses, i.e., stunting, epinasty, chlorotic or necrotic spots, malformation of flower shape, flower color bleaching, poor root growth, and loss of plants (Kovalskaya and Hammond, 2014). Thus, it can be speculated that the basic mechanisms involved in the pathogenicity of both viruses and viroids are similar. However, plant viruses have more than 5 viral origin proteins that induce pathological effects, whereas viroids have no coding protein (Gómez et al., 2008). Accordingly, their replication and interactions with host factors have been intensively studied. Viroids of *Pospiviroidae* and *Avsunviroidae* replicate in the nucleus and chloroplasts of the host cell, respectively, through an RNA-based rolling circle mechanism, but the former is in an asymmetric manner and the latter is in a symmetric manner (Nohales et al., 2012). The infected circular (+) monomer is replicated into linear (-) concatemeric strands which are then cut and ligated, eventually producing a (-) monomer in a symmetric model. Using this (-) monomer as a template, the same steps are repeated to produce (+) monomer (Fig. 1A). On the other hand, in an asymmetric...
model, linear (-) concatemeric strands replicated from a circu-
lar (+) monomer are used to produce a linear (+) concatemeric
strand which is directly cut and ligated as a (+) monomer (Fig.
1B). A member of Pospivirodase replicates in the nucleus us-
ing a host DNA-dependent RNA polymerase II (Diner, 1986),
suggesting that disruption of the host transcription system by
viroid RNA multiplication is involved in the pathogenic symp-
toms of infected plants. Proteomic analysis of Citrus exocortis
viroid (CEVd)-infected tomato plants has shown that differen-
tial expressions of defense proteins, such as an endochitinase,
and pathogenesis-related proteins, such as β-glucanase, were
induced, whereas constant expressions were maintained in ribo-
somal protein and translational factors, such as elongation and
translation initiation factors (Lisón et al., 2013). The binding
of viroid RNA to host factors has been reported in some mem-
bers of Pospiviroidae, e.g., phloem lectin protein PP2 binds
to Hop stunt viroid (HSVd) (Gómez and Pallás, 2004), a bro-
modomain-containing Virp1 protein that specifically interacts
with Potato spindle tuber viroid (PSTVd) (+) RNA (Martínez
et al., 2003; Kalantidis et al., 2007). On the other hand, using
the northwestern hybridization technique, Dubé et al. (2009)
showed that a Peach latent viroid (PMLVd) of Avsunviroidae
binds to elongation factor a-1 (eEF1A), which is involved in
diverse cellular mechanisms including translation, cytoskeleton
formation, and protein export. The structural domain of viroid
RNA binding to the host factors mimics those of cellular func-
tional RNA in plants, and it is reasonable to speculate that viroid
RNA changes host enzymes and other components indispensable
for normal biochemical and physiological cellular processes.
Although accumulated evidence has allowed speculations or
discussions of viroid pathogenesis, clear evidence connecting
the presence of viroid RNA and macroscopic symptoms in in-
fected plants is limited. In this context, another possibility that
post-transcriptional gene silencing in infected plants is related to
viroid pathogenicity was proposed (Wang et al., 2004).

Small interfering RNAs (siRNA) induces gene silencing

A general scheme of siRNA generation and the process of gene
silencing are summarized in Fig. 2. It is speculated that siRNA
was primitively developed as an immune response to invasion by
foreign nucleic acids. siRNA is 21-30 base pairs of double-strand
RNA (dsRNA) which is produced by ribonuclease Dicer protein
(also known as Dicer like-protein (DCL) in plants) from long
dsRNA that is not usually found in cells. This primary siRNA is
used as a template, amplifying the secondary siRNA with the aid
of RNA-dependent RNA polymerase (RDR). This amplification
mechanism allows abundant siRNA generation, eventually in-
ducing a more intense silencing of the target gene. Either strand
of siRNA is incorporated as a guide strand into the RNA-induced
silencing complex (RISC) with Argonaute proteins (AGOs). The
guide strand in RISC pairs with a complementary sequence of
target mRNA and cleaves it through the action of AGOs, eventu-
ally inducing post-transcriptional gene silencing. Furthermore,
siRNA is also used to direct the de novo DNA methylation of the
original genes that produce RNA, and this transcriptional gene
silencing is suggested to be a defense mechanism against virus infection and/or transposons.

Since their intramolecular base paired secondary structure and dsRNA formation during replication give DCL a chance to recognize these molecules as target substrates, viroids generate siRNA of their own sequence. Early evidence that viroids induce gene silencing was reported by Wasseneeger et al. (1994), whereby a PSTVd sequence incorporated into a transgenic tobacco genome was methylated by the over-expression of PSTVd RNA. Using PSTVd-infected tomato plants, Itaya et al. (2001) reported that there was quantitative relationship among the level of siRNA, level of PSTVd accumulation, and development of symptoms. Wang et al. (2004) showed that PSTVd and cucumber mosaic virus satellite pathogenecities were mediated by RNA silencing; a pathogenic symptom of tobacco caused by Y satellite (parasite RNA harbored in virus particles, containing a high degree of secondary structural similarity to viroids) of cucumber mosaic virus was markedly reduced by suppressing RNA silencing using the potent suppressor He-Pro; expression of hairpin RNA derived from PSTVd in tomato led to symptoms similar to those of PSTVd infection. Gómez et al. (2008), using a grafting technique with *Nicotiana benthamiana*, showed that RDR 6, which plays a role in secondary siRNA initiation, was required for the development of HSVd symptoms. In this case, the scion of *rdr 6* lacking RDR6 activity or wild-type was grafted onto HSVd-infected stock, and the amount of HSVd RNA and symptom development were evaluated. Although HSVd RNA accumulated in both scions, no symptom was observed in the *rdr6* scion, whereas marked symptoms were detected in the wild-type scion. Tsuro et al. (2013) detected fluctuations of CSVd siRNA in infected chrysanthemums cultured from 5 to 28°C. Under low temperature conditions (5°C), stunted growth, the most prominent symptom of CSVd infection, was relieved, and siRNA was lowered to undetectable level. However, an intermediate temperature (22°C) increased the maximum siRNA level and induced markedly stunted growth compared to the uninfected control. These results suggest that viroid RNA can cause the silencing of endogenous genes normally expressed in the host plant. Besides the induction of the post-transcriptional gene silencing, viroid RNA may interact with host enzymes involved in the RNA-directed DNA methylation pathway, which is important in transcriptional gene silencing (Navarro et al., 2009). More recently, direct evidence of specific mRNA cleavage targeted by viroid siRNA was reported; mRNA encoding chloroplastic heat-shock protein 90 (cHSP90) in *Prunus persica* was targeted and degraded by siRNA of *Peach latent mosaic viroid* (PLMVd), eventually causing albimism, a typical symptom of PLMVd infection, since cHSP90 is involved in chloroplast biogenesis and plastid-to-nucleus signal transduction (Navarro et al., 2012). Taken together, the evidence suggests that viroids generate siRNA of their own sequence and this transcriptionally and/or post-transcriptionally suppresses the expression of endogenous genes that are indispensable for the normal growth and development of plants.

**Our approach toward CSVd-resistant breeding of chrysanthemum**

Conventional plant breeding methods to improve CSVd resistance using the useful genetic resources is the most valuable approach (Nabeshima et al., 2012, 2014), whereas the molecular approach is also effective, since the genome of the pathogen only consists of RNA. Accordingly, an application of ribozymes that specifically cleaved the CSVd RNA sequence was our initial approach (Ando et al., 2006). However, since ribozymes require both high temperature and high salt conditions to maximize their efficiency to cleave target RNA, ribozyme activities *in planta* are not sufficient to reduce CSVd RNA, even though high-level expression of ribozyme genes can be achieved in transgenic chrysanthemum. Thus, we considered whether siRNA of CSVd targets and cleaves its own sequence.

Plant viruses develop defense mechanisms to counter host resistance by producing the p19 protein that directly binds to siRNA targeting the viral RNA genome (Várallyay et al., 2014). Although viroids have no such proteins, several lines of evidence suggest that viroid RNA is free from silencing mechanisms due to their (1) highly ordered secondary structure, (2) association with host factors, (3) subcellular localization, and (4) unknown novel suppressor (Kovalskaya and Hammond, 2014). However, some evidence for siRNA mediating viroid resistance was obtained: artificially designed hairpin RNA (hpRNA)-derived siRNA of PSTVd appeared to effectively target the mature PSTVd RNA, eventually preventing PSTVd replication in tomato (Schwind et al., 2009; the accumulation of PSTVd in the scion of *N. benthamiana* was attenuated when grafted to transgenic *N. benthamiana* stock expressing hpRNA of PSTVd (Kasai et al., 2013). These results support using the siRNA technique for the molecular breeding of CSVd-resistant chrysanthemum cultivars.

To test if CSVd-siRNA targets and cleaves CSVd RNA, Yanagimoto (2010) constructed the *luciferase* (*luc*) reporter gene in which the head to head dimer of the CSVd sequence was inserted between *luc* ORF and the nopaline synthase (NOS) terminator in the Ti-plasmid vector (pIL-Csvd) (Fig. 3A), and carried out a transit expression assay by agroinfiltration in the leaves of CSVd-infected or healthy chrysanthemum plants. In the case of CSVd-siRNA cleaving the CSVd sequence of the reporter mRNA, it was expected that activities of luc tend to be lower than in healthy plants. As shown in Fig. 3B, this was true, while no such decline was observed in the *luc* reporter gene lacking CSVd sequence (pIL), suggesting that naturally generated siRNA from
CSVd RNA targets and cleaves its own RNA sequence. Finally, we have developed Ti-plasmid constructs expressing hpRNA of some parts of the CSVd genome and developed a transgenic chrysanthemum plant expressing siRNA of CSVd. The analysis of the CSVd resistance of these plants is ongoing.

References


キクのわい化ウイロイド（CSVd）病抵抗性の向上にむけての研究

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要旨：ウイロイドは最小の RNA 病原体で、園芸作物ならびに農作物に著しい被害をもたらす。ウイロイドはタンパク質をコードする遺伝子を持たず、感染した植物体内での複製と移動は宿主細胞の諸因子に依存する。しかし、ウイロイドにより病状が現れる機構は不明な点が多い。近年、ウイロイドによる病状が現れる機構を不変化する short interfering RNA (siRNA) が宿主遺伝子の発現を抑圧し、これが病状抑圧につながるとする説が有力になりつつある。ウイロイドの生物学についての最近の知見を簡潔にまとめてると共に、キクわい化ウイロイド（CSVd）病抵抗性育種に向けての我々の取り組みについて報告する。

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