Research Note

Potato spindle tuber viroid as Inducer of RNA Silencing in Infected Tomato

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Potato spindle tuber viroid (PSTVd), an RNA plant pathogen encoding no known proteins, induces systemic symptoms on tomato plants. We report detection of small RNAs of approximately 25 nucleotides with sequence specificity to PSTVd in infected plants: an indication of the presence of RNA silencing. RNA silencing, however, did not appear to be responsible for the differing symptoms induced by a mild and a severe strain of PSTVd. The unique structural and biological features of viroids make them attractive experimental tools to investigate mechanisms of RNA silencing and pathogen counterdefense.

Additional keywords: gene silencing.

RNA silencing is an important mechanism of gene regulation in many organisms, including plants (Bass 2000; Carthew 2001; Lindbo et al. 2001; Matzke et al. 2001; Vaucheret and Fagard 2001). RNA silencing can be subclassified into RNA-mediated transcriptional gene silencing (TGS) and posttranscriptional gene silencing (PTGS). RNA-mediated TGS occurs when double-stranded (ds) RNA 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). PTGS, on the other hand, (i) yields reduced steady state levels of targeted host or viral cytoplasmic RNA, and to a lesser, but sometimes observable, reduction of nuclear RNA (Lindbo et al. 1993; Montgomery et al. 1998; van Blockland et al. 1994); and (ii) may be mediated through a host-encoded RNA-dependent RNA polymerase (RdRp) and RNA helicase (Cogoni and Macino 1999; Dalmay et al. 2000, 2001; Domeier et al. 2000; Mourrain et al. 2000). A hallmark of RNA-mediated TGS and PTGS is the production of small RNAs of 21 to 23 nucleotides (nt) with sequence specific to the silenced gene (Elbashir et al. 2001; Hamilton and Baulcombe 1999; Mette et al. 2000; Zamore et al. 2000). A plant virus can be both the inducer and target of RNA silencing (Baulcombe 1996; Marathe et al. 2000). RNA silencing is, therefore, believed to

play a role in host defense against pathogen infection, as well as in inactivating expression of undesired host genes (Matzke et al. 2001).

Viroids are single-stranded, covalently closed, circular RNA molecules 246 to 499 nt long (Mathews 1991). In striking contrast to viruses, viroids do not encode any proteins and are not known to be encapsidated (Mathews 1991). Wassenegger et al. (1994) demonstrated that *Potato spindle tuber viroid* (PSTVd) can trigger methylation of PSTVd cDNA sequences stably incorporated into the transgenic tobacco genome. Interestingly, a DNA target of 30 bp is sufficient for this methylation to occur (Pelissier and Wassenegger 2000). The size of the target is similar to the size of the small RNAs produced during RNA-mediated TGS and PTGS. Thus, viroid infection may be a unique system to study RNA silencing, but more information is necessary to establish this experimental system. For example, it has not been reported whether infections of plant tissue with viroids yield the small RNAs associated with RNA silencing. Given that RNA silencing is a defense mechanism against viral infection, it is also of outstanding interest to determine whether RNA silencing could contribute to the varying symptoms elicited by different viroid strains.

To test whether PSTVd could trigger the production of small RNAs, we analyzed PSTVd-infected tomato plants for the presence of small RNAs with sequence specificity to PSTVd. We inoculated cotyledons of 10-day-old tomato plants (*Lycopersicon esculentum* cv. Rutgers) with in vitro transcripts of two strains of PSTVd, the mild and RG1 (severe) (Owens et al. 1996). Forty nanograms of RNA was used to inoculate each plant. Diethylpyrocarbonate-treated water was used as the mock inoculum. The plants were grown in a growth chamber set for an 18-h-light (26°C)/6-h-dark (26°C) cycle. RG1-infected plants started showing symptoms at 17 days postinoculation (dpi) and mild-strain-infected plants started showing symptoms at 25 dpi. Samples from systemic leaves were collected from all inoculated plants at 17 and 25 dpi. Total RNA was isolated from the systemic leaves and PSTVd-specific small RNA was detected essentially as described by Hamilton and Baulcombe (1999), except that total RNA without enrichment of small RNA was loaded on the gel (Hutvágner et al. 2000). Briefly, the RNA samples were separated by electrophoresis through a 15%-polyacrylamide–8-M-urea gel, transferred onto a nylon

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membrane, and cross-linked by UV. A 25-nt DNA oligonucleotide was loaded on the same gel as a size marker. The blotted membrane was prehybridized at 42°C for 30 min and then hybridized with an α -³²P UTP-labeled, minus-strand (–) PSTVd RNA probe overnight at 35°C. The intensity of each individual band was determined by phosphor-imager scanning, and the relative amounts of RNA were quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

We detected small RNAs of approximately 25 nt in both mild-strain- and RG1-infected tomato plants, but not in mock-inoculated plants (Fig. 1 and data not shown). The amount of small RNA was greater in RG1-infected plants than in mild-strain-infected plants (approximately 1.4- and 1.9-fold at 17 and 25 dpi, respectively; values represent the mean of three independent experiments). For both strains, the amount of small RNA was higher at 25 dpi than at 17 dpi (2.5- and 3.5-fold higher for mild and RG1 strains, respectively). Also for both strains, the amount of viroid was higher at 25 dpi than at 17 dpi (1.5- and 4.0-fold higher for mild and RG1 strains, respectively; values represent the mean for three independent experiments). Thus, the amount of small RNA and viroid increased with time for each strain. In light of the consistent production of small RNAs in all other RNA-silencing systems, we interpret the presence of PSTVd-specific small RNA in the infected tomato plants as an indication of viroid-induced RNA silencing (VdIRS).

Current models suggest that elicitors of RNA silencing include dsRNAs that are formed during replication of RNA viruses or that are formed from aberrant host RNA templates by an RdRp (Lindbo et al. 2001; Matzke et al. 2001). Biochemical studies employing *Drosophila* spp. have provided some mechanistic insights into this process. Based on these studies, substrate dsRNAs are cleaved into 21- to 23-nt small RNAs by an RNase III-like nuclease (Bernstein et al. 2001). Strikingly, a target mRNA is cleaved at 21 to 23 intervals, suggesting that the small RNAs derived from the dsRNAs serve as a guide to RNAs targeted for mRNA degradation (Elbashir et al. 2001; Zamore et al. 2000). dsRNAs of greater than 38 nt are necessary to potentiate PTGS (Elbashir et al. 2001).

PSTVd replication involves a rolling-circle mechanism (Branch and Robertson 1984). In this mechanism, the plus-strand (+), circular viroid molecule serves as the initial template for the production of a linear and multimeric (–) RNA. This (–) RNA is then used as the template for the synthesis of a linear and multimeric (+) RNA. The (+) RNA is subsequently cleaved into unit-length RNAs that are circularized to form the mature viroid molecules. Based on these findings, dsRNAs are transiently formed at several stages of PSTVd replication and they could be the target of an RNase III-like nuclease. The observed 25-nt RNAs could be a mixture of the dsRNA derivatives and the target RNA degradation products.

Pelissier and Wassenegger (2000) suggested that the full-size, mature PSTVd molecules function to induce DNA methylation. In light of our detection of PSTVd-specific small RNAs in the infected tomato, we suggest that such small RNAs could be the alternative RNA forms that induce DNA methylation. This model, consistent with the general idea

proposed by Matzke et al. (2001), is attractive because it could explain how a DNA target of 30 bp is sufficient for methylation to occur (Pelissier and Wassenegger 2000). Further studies on VdIRS could provide a critical test of the model.

How does PSTVd evade host gene silencing? Several possibilities can be considered. First, if PSTVd exists primarily as a rod-shaped structure in the cell during its life cycle, extensive base-pairing within the molecule would form a complex secondary structure that prevents recognition by the small RNA guides. The accepted secondary structure does not contain any dsRNA stretches of 23 nt or greater in length; therefore, the final form of the viroid would be an unlikely target for an RNase III-like nuclease. Second, viroids may be bound to cellular proteins to be protected. Third, a viroid may actively suppress RNA silencing either by activating a host protein suppressor of RNA silencing or by directly silencing host genes in the pathway. A recent study identified a calmodulinlike plant protein that functions

Fig. 1. Upper panels show detection of small RNAs in *Potato spindle tuber viroid* (PSTVd)-infected tomato plants. Total RNA from indicated plants was isolated using Trizol reagent (Gibco-BRL, Rockville, MD, U.S.A.) and 80 µg of total RNA was loaded in each lane. For a size marker, 25-nt PSTVd oligonucleotides were loaded on the same gel (upper left panel). The blotted membrane was prehybridized in ULTR Hyb (Ambion, Austin, TX, U.S.A.) at 42°C for 30 min and then hybridized in the same solution with a PSTVd minus-strand (–) RNA probe labeled with α -³²P UTP at 35°C overnight. Small RNAs were detected in mild-strain- and RG1-infected plants, but not in mock-inoculated plants (upper right panel). The signal above the 25-nt oligomer came from the RNA probe hybridized to the PSTVd plus-strand (+) RNA that was originally loaded in the adjacent lane as a positive control, but was cut out for imaging purpose. Consistent with Hamilton and Baulcombe's (1999) observations, some nonspecific probe binding (for instance bands in the lanes containing the sample from mock-inoculated plants) occurs at the 35°C hybridization temperature, which is necessary to detect the small RNA. Such nonspecific binding is eliminated when hybridization is conducted at 55°C, which, however, leads to poor detection of the small RNA. The bottom panel shows detection of PSTVd under 55°C prehybridization and hybridization conditions with reduced background signals. All lanes in this panel contain the same types of samples as indicated in the upper right panel. Total RNA (40 µg) was loaded in each lane.

as a suppressor of PTGS (Anandalakshmi et al. 2000). Thus, there is an endogenous mechanism with which PSTVd could potentially interact to suppress PTGS. It will be interesting to determine if this host protein is induced during PSTVd infection.

If an increase in the amount of the small RNA is indicative of enhanced RNA silencing, one would expect that the level of PSTVd accumulation and, therefore, symptom severity decrease accordingly. However, this was not observed. Rather, the level of small RNA increased with increased PSTVd accumulation levels and development of symptoms. The results provide support for the hypothesis that selective molecular interactions of different viroid strains with host factors contribute to varying degrees of symptom expression (Owens et al. 1996).

In summary, we have obtained evidence that PSTVd can be an activator and target of RNA silencing. However, the differing symptom severities induced by the two viroid strains were positively correlated with the level of small RNAs produced, indicating that RNA silencing was unlikely to be responsible for the differing disease phenotypes. The many unique features of PSTVd make it a potentially useful tool to investigate the mechanism of RNA silencing, as well as any counterdefenses the pathogen may display.

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NOTE ADDED IN PROOF

While this manuscript was under review, a paper (Papaefthimiou et al. 2001) was published describing results similar to those reported here.

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