Viroids are small, highly base-paired, covalently closed circular, single-stranded pathogenic RNAs. Following the 1971 discovery of potato spindle tuber viroid (PSTVd), almost 30 additional species of this novel class of autonomously replicating pathogens have been described (Hadidi et al., 2003). Unlike RNA viruses, the genetic information encoded by PSTVd and other viroids is not translated into protein, and viroid replication is completely dependent on the host’s transcriptional machinery (Flores et al., 2005; Ding & Itaya, 2007).

As might be expected from their highly base-paired structure and RNA–RNA mode of replication, viroids have been shown to induce RNA silencing. Accumulation of viroid-specific small RNA (Vd-sRNA) was first detected in tomato plants infected with PSTVd (Itaya et al., 2001; Papaefthimiou et al., 2001). Later, three viroids belonging to the family *Avsunviroidae*, that replicate in the chloroplast rather than the nucleus, were also reported to induce RNA silencing (Martinez de Alba et al., 2002; Markarian et al., 2004). Vd-sRNA appears to play an important role in mediating viroid symptom expression (Markarian et al., 2004; Wang et al., 2004).

Previous studies from our laboratory have suggested that RNA silencing could be responsible for the recovery phenomenon observed in PSTVd-infected tomato during the later stages of infection (Sano & Matsuura, 2004). To examine the relationship between RNA silencing and recovery in greater detail, we have carried out time-course analyses of viroid-specific small RNA accumulation using several viroid–host combinations. These analyses revealed the presence of two size classes of viroid-specific small RNAs in infected plants, and sequence analysis subsequently demonstrated the presence of a previously undescribed cluster of small RNAs derived primarily from negative-strand PSTVd RNA. Although the clustering patterns were similar, the size distribution of PSTVd small RNAs isolated from symptomatic leaf tissue became more heterogeneous with time. The process by which viroid-specific small RNAs are generated appears to be more complicated than previously believed, possibly involving multiple DICER-LIKE activities, viroid RNA substrates and subcellular compartments.
PSTVd progeny and srPSTVds at later times, i.e. between 14 and 45 d.p.i.

Faint PSTVd signals were first observed in leaves 3–5 at 12 d.p.i., but the concentration of srPSTVds remained below detectable levels (data not shown). Two days later, as primary leaf curling first began to appear in leaf 3, results of Northern analyses shown in Fig. 1(a) revealed the presence of detectable concentrations of srPSTVd in leaves 1–5. At 16 d.p.i., as symptoms of epinasty and stunting began to intensify, data shown in Fig. 1(b) revealed that PSTVd accumulation also increased, reaching maximum levels in leaves 3–10 on day 18 and maintaining these levels until sometime between days 30 and 45. As leaves 9–10

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**Fig. 1.** Time-dependent accumulation of srPSTVds (a) and PSTVd progeny (b) in infected tomato leaves. Aliquots (2 μg) of RNAs were fractionated in 12% polyacrylamide (a) or 1.5% agarose (b) gels, transferred to nylon membranes and hybridized with a DIG-labelled PSTVd cRNA. Numbers beneath each lane indicate leaf number. Numbers ending with ‘d’ indicate leaves showing disease symptoms; those ending in ‘r’ indicate leaves exhibiting recovery; Fb, flower bud. Arrows denote positions of 20 nt and 24 nt PSTVd positive strand-specific DNA markers. As a loading control, the 4S-5S RNA region of each gel was stained with ethidium bromide. The positive control contains serial 5-fold dilutions of purified PSTVd (0.03–20 ng per lane). Relative concentrations of PSTVd progeny and two size classes of srPSTVd were estimated by Northern analysis over the two-week period when visible symptoms just begin to appear and then intensify. (c) Schematic semi-quantitative summary of the data presented in Figs 1(a) and 1(b). Note that the smaller srPSTVds consistently appear two or more days before the larger ones.
reached full expansion between 30 and 45 d.p.i., symptom severity decreased dramatically.

As shown in Fig. 1(a), older, fully expanded leaves contained two distinct size classes of srPSTVd, i.e. molecules containing 21–22 nt and ca 24 nt. This is most clearly seen for leaves 2–4 (14 d.p.i.), leaves 2–6 (16 d.p.i.), leaves 1–7 (18 d.p.i.) and leaves 3–10 (25 d.p.i.). In contrast, only shorter srPSTVd (ca 21 nt) were visible in the upper expanding leaves of PSTVd-infected tomato, i.e. leaf 5 (14 d.p.i.), leaf 7 (16 d.p.i.), leaves 8–9 and the flower bud (18 d.p.i.), and leaf 10 (25 d.p.i.). The result indicates that at 14–18 d.p.i. just after the induction of RNA silencing, only short srPSTVd was detectable in the leaf primordia near the shoot apex; in contrast, both short and long molecules were clearly detectable in the fully expanded, lower leaves showing prominent disease symptoms. When the Northern analysis data were replotted in a schematic, semiquantitative fashion (see Fig. 1c), accumulation of longer srPSTVd could be seen to consistently lag 2–3 days behind that of the shorter species (Fig. 1c). Late in infection (i.e. 30–45 d.p.i.), srPSTVd became more heterogeneous in size.

To get a clearer understanding of srPSTVd accumulation in the uppermost leaves and shoot apex, a second experiment was performed (Fig. 2a). Pooled samples from leaves 1–9 and the apical meristem were assembled from 50 plants at 15 d.p.i. and analysed for the presence of srPSTVds. Consistent with the pattern seen in the first experiment (see Fig. 1a; 14 and 16 d.p.i.), leaves 6–9 appeared to contain only the smaller class of srPSTVd. Two size classes of srPSTVd were once again visible in the lower, fully expanded leaves 2–4 (data not shown; results similar to those shown in Fig. 1a). It would appear that once RNA silencing is triggered in PSTVd-infected plants, short srPSTVd containing ca 21 nt quickly accumulates to detectable levels in all above-ground portions of the plant, except the apical meristem.

To determine how general the presence of these two size classes of Vd-sRNAs in infected tissue might be, we next examined four other viroid–host combinations, i.e. cucumber infected with hop stunt viroid (HSVd) and hops infected with hop latent viroid (HLVd), HLVd+HSVd, or HLVd+apple fruit crinkle viroid (AFCVd). Leaf tissue harvested from tomatoes infected with cucumber mosaic virus (CMV), where small RNA synthesis presumably occurs in the cytoplasm rather than the nucleus, was included as a control.

The time-course of symptom development and recovery for cucumber seedlings inoculated with HSVd at the cotyledon stage was very similar to that observed for PSTVd-infected tomato. Thirty d.p.i., pooled tissue samples from leaves 1–18 were assembled from ten plants and analysed for the presence of srHSVds. As shown in Fig. 2(b), the older portions of the cucumber plants (leaves 4–14) contained two different sizes of srHSVds; in the youngest leaves, the upper band was very faint. Overall, the distribution pattern of these two size classes of srHSVd was quite similar to that observed for srPSTVd in tomato.

Field-grown hops infected with either HLVd alone or mixtures of HLVd+HSVd or HLVd+AFCVd were also examined for the presence of the respective Vd-sRNAs. As shown in Fig. 2(c), small RNAs derived from all three viroids were easily detectable in mature hops. Comparison of the left and right panels reveals that infection with either HSVd or HLVd led to the accumulation of two size classes of Vd-sRNA. In contrast, plants infected with HLVd+AFCVd contained relatively little of the longer srAFCVd (middle panel). Although it was not possible to follow
accumulation patterns under field conditions in detail, at least two out of three viroids infecting hops appeared to give rise to two classes of small RNA. For purposes of comparison, we also examined the size class of small RNAs associated with CMV, a tripartite RNA virus replicating in the cytoplasm. Data presented in Fig. 2(d) show that, under the same experimental conditions used for PSTVd-infected plants, fully expanded lower leaves contained only short small RNA 14 d.p.i. No longer small RNAs were detectable.

As described in the Supplementary Materials, available with the online version of this paper, cDNAs derived from electrophoretically purified srPSTVds were amplified, hybrid-selected using biotinylated PSTVd negative-strand RNA as capture probe (Tschudi et al., 2003), and cloned for sequence analysis. Purified srPSTVds containing shorter and longer size classes of small RNAs were prepared from the first symptomatic leaf (i.e. leaf 3) of tomato plants harvested approximately one week after symptoms had begun to appear (i.e. 20 d.p.i.). A comparable preparation of srPSTVd from the still-unfolding leaf 8 was also analysed.

As shown in Fig. 3(a), the 89 srPSTVd sequences recovered from leaf 3 were derived from the left and right terminal domains plus the border between the lower portions of the variable and central domains. In contrast to the results recently reported by Itaya et al. (2007), many srPSTVds were derived from PSTVd negative-strand RNA. Such srPSTVds were particularly common among the molecules originating from the border between the variable and central domains. A similar clustering was observed for the 85 srPSTVds recovered from leaf 8 (results not shown).

The histograms shown in Fig. 3(b) compare the length distributions for the srPSTVd populations recovered from leaves 3 and 8. Data for srPSTVds derived from both the positive- and negative-strand PSTVd RNAs are presented, and two conclusions are immediately apparent. First, the srPSTVds recovered from leaf 8 appear to be more homogeneous in length than those from leaf 3; second, comparatively few 24 nt srPSTVds were recovered from either sample. Taken together, these results suggest that the mechanism responsible for srPSTVds biogenesis may well be more complicated than previously believed.

Following the establishment of PSTVd-induced RNA silencing in tomato plants, two size classes of srPSTVds were detectable. Shorter molecules containing ca 21 nt were the first to appear, and longer ones ca 24 nt in length appeared several days later. A similar pattern was observed in cucumber plants infected with HSVd and field-grown hops infected with HLVd or doubly infected with HLVd plus HSVd or AFCVd. Analysis of leaf tissue collected from CMV-infected tomato, in contrast, revealed quite a different picture. No long srCMV could be detected in any of the leaves, even in fully expanded symptomatic leaves, and thus, the pattern of small RNA accumulation in which synthesis of short 21–22 nt small RNAs is followed several days later by the synthesis of longer molecules containing ca 24 nt appears to be unique to viroids that replicate in the host cell nucleus.

This chronology suggests a possible process by which RNA silencing reduces the severity of PSTVd disease while leaving replication relatively unaffected; namely, (i) immediately after induction of silencing, srPSTVd spreads systemically in the plant, (ii) leaf primordia developing thereafter are exposed to the RNA silencing from the beginning of the differentiation process and (iii) a marked suppression of visible disease becomes visible in the upper portions of infected plants as these leaves expand over the next two weeks. Even though PSTVd titres are somewhat lower in leaves exhibiting this recovery phenomenon (Sano & Matsuura, 2004), PSTVd accumulation remains relatively high up to 30 d.p.i. Unlike potato virus X and several other RNA viruses in which the onset of RNA silencing leads to a dramatic drop in viral RNA accumulation, PSTVd replication appears to be relatively resistant to RNA silencing (Wang et al., 2004; Itaya et al., 2007).

Sequence analysis of srPSTVd populations isolated from tomato leaves of different ages indicated that more than half of such molecules were derived from non-genomic PSTVd RNA(s), either from double-stranded molecules...
such as replicative intermediates or single-stranded negative-strand RNA. Both positive- and negative-strand PSTVd RNAs are known to assume highly base-paired dsRNA-like conformations (e.g. Steger & Riesner, 2003), and it is likely that either could serve as substrate for srPSTVd synthesis, as has been reported for the genomic RNA of certain plant viruses (Molnár et al., 2005; Ho et al., 2006). Indeed, Itaya et al. (2007) have recently presented evidence that (i) srPSTVd are predominantly derived from the positive-strand genomic RNA and (ii) incubation of full-length, linear positive-strand PSTVd RNA with a partially purified preparation of DICER-LIKE enzymes (DCLs) from Arabidopsis releases small RNAs containing ~21 nt. Significantly, two of the hotspots for srPSTVd biogenesis revealed by our analyses (i.e. those in the right and left terminal domains) are the same as those reported by Itaya et al. (2007).

The most striking difference between our respective datasets involves a third srPSTVd hotspot located between the central and variable domains on the lower strand of PSTVd. Here, a large majority of srPSTVd was derived from the negative rather than the positive strand. More extensive sequencing studies will be required to completely resolve this apparent discrepancy in srPSTVd origin, but we note that interaction of nearby positions 227–236 and 319–328 results in the formation of so-called ‘secondary hairpin II’. Mutational analysis of PSTVd secondary hairpin II suggests that it plays a critical role in negative-strand function in vivo (Loss et al., 1991), and perhaps one or more DCLs also recognize this alternative structural feature. It is also possible that sampling differences may be responsible for some of the differences observed. For example, our srPSTVd samples were extracted from still-expanding leaves 20 d.p.i., while Itaya et al. (2007) used a combination of new and expanding leaves collected 25–30 d.p.i. (Asuka Itaya, personal communication). As noted above, srPSTVd accumulation patterns were seen to differ dramatically depending on the stage of leaf development, and evidence was obtained for a time-dependent increase in srPSTVd size.

According to currently accepted models for small RNA biogenesis (e.g. Tang et al., 2003; Hamilton et al., 2002; Papp et al., 2003; Vaucheret 2006), different DCLs are involved in the synthesis of different size classes of small RNAs. The fact that plants infected with pospiviroids contain both long and short small RNAs strongly suggests the involvement of two or more DCLs. It is possible that PSTVd replicative intermediates located in the nucleus are processed by the tomato counterparts of DCL-1, -3 or -4 into small 19–22 nt RNAs which are then exported into the cytoplasm (Denti et al., 2004) via pathway(s) involving HASTY and/or other nucleocytoplasmic transport receptors (Park et al., 2005). Later, the large amounts of PSTVd progeny which accumulate in the nucleus/nucleolus could be processed by DCL-3 (or another DCL) to produce longer srPSTVd. Xie et al. (2004) have shown that DCL-3 is localized in the nucleus, and several other reports have shown that knocking out DCL-3 abolishes the synthesis of 24 nt small RNA in Arabidopsis (reviewed by Herr, 2005; Qi & Hannon, 2005). According to this scenario, the failure of CMV to enter the nucleus would protect it against degradation by DCL-3, thereby blocking the synthesis of longer small RNAs.

A final level of complexity may be due to the terminal methylation that is commonly observed for plant small RNA species (Yu et al., 2005; Ebhardt et al., 2005; Yang et al., 2006). In our analyses, the cloning efficiency of longer srPSTVd containing 23–24 nt was quite low, in spite of the intense hybridization signals observed in Northern analysis. Recently, Pak & Fire (2007) and Sijen et al. (2007) have shown that the secondary short interfering RNAs produced by RNA-directed RNA polymerases in Caenorhabditis elegans contain 5’-di- or triphosphate termini, resulting in a dramatic decrease in cloning efficiency. Pak & Fire (2007) further noted that (i) the cloning efficiency of small antisense RNAs could be increased by using a 5’-ligation-independent cloning method and (ii) small antisense RNAs cloned with a 5’-ligation-independent method were 1 nt longer than those cloned with a 5’-ligation-dependent method. Since our studies relied on a 5’-ligation-dependent method, the lower cloning efficiency of longer srPSTVd could be explained by the cloning method used. The longer srPSTVds observed in this research could represent secondary small RNAs with similar di- or triphosphate termini.

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References


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