RNAi-mediated resistance to *Potato spindle tuber viroid* in transgenic tomato expressing a viroid hairpin RNA construct

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SUMMARY

Because of their highly ordered structure, mature viroid RNA molecules are assumed to be resistant to degradation by RNA interference (RNAi). In this article, we report that transgenic tomato plants expressing a hairpin RNA (hpRNA) construct derived from *Potato spindle tuber viroid* (PSTVd) sequences exhibit resistance to PSTVd infection. Resistance seems to be correlated with high-level accumulation of hpRNA-derived short interfering RNAs (siRNAs) in the plant. Thus, although small RNAs produced by infecting viroids [small RNAs of PSTVd (srPSTVds)] do not silence viroid RNAs efficiently to prevent their replication, hpRNA-derived siRNAs (hp-siRNAs) appear to effectively target the mature viroid RNA. Genomic mapping of the hp-siRNAs revealed an unequal distribution of 21- and 24-nucleotide siRNAs of both (+)- and (–)-strand polarities along the PSTVd genome. These data suggest that RNAi can be employed to engineer plants for viroid resistance, as has been well established for viruses.

INTRODUCTION

Viroids are non-encapsidated, single-stranded (ss), 250–400-nucleotide-long circular RNA molecules that do not encode proteins. They exclusively infect plants and autonomously replicate via the RNA/RNA pathway in the nucleus (*Pospiviroidae*) or chloroplast (*Avsunviroidae*) (Ding and Itaya, 2007; Flores *et al.*, 2005; Tabler and Tsagris, 2004). Viroids in the family *Pospiviroidae* have a wide host range, including crop and ornamental plants. The type member of *Pospiviroidae* is the *Potato spindle tuber viroid* (PSTVd), more than 130 variants of which have been characterized (Rocheleau and Pelchat, 2006).

In Europe, the USA and Australia, PSTVd is classified as a quarantine pathogen. Infection of potato and tomato plants can lead to massive crop losses. Since late 2006, an epidemic outbreak of PSTVd in the ornamental plants *Solanum jasminoides*, *S. rantonnetii* and *Brugmansia* spp. has been reported in the Netherlands, Germany and Italy (Di Serio, 2007; Verhoeven *et al.*, 2008). In contrast with tomato and potato, these ornamental plants do not develop visible disease symptoms with PSTVd infection. Unfortunately, infected plants were already widely distributed throughout Europe before viroid detection. Since transmission to crop plants is always a potential threat and PSTVd-resistant tomato and potato plants are not available, the production and distribution of ornamental PSTVd host plant species have been restricted. This restriction has a severe economic impact on European plant producers, arguing for the availability of PSTVd-resistant crop plants.

In plants, viral and viroid infections activate an RNA interference (RNAi)-mediated defence mechanism that involves the accumulation of short interfering RNAs (siRNAs) corresponding to the genome of an infecting pathogen (Itaya *et al.*, 2001; Lecellier and Voinnet 2004; Papaefthimiou *et al.*, 2001; Voinnet, 2001). However, these siRNAs are obviously unable to prevent pathogen attack because viruses and viroids can still systemically infect their host. At least for viroids, it has been reported that PSTVd only replicates in approximately 20% of the leaf cells (Harders *et al.*, 1989). The possible absence or low levels of siRNAs in non-PSTVd-replicating cells could provide a refuge for the viroids to avoid RNA silencing-mediated plant defence. RNAi-based strategies have been successfully applied to the development of plants resistant to numerous viruses (Chen *et al.*, 2004; Hily *et al.*, 2005; Kalantidis *et al.*, 2002; Niu *et al.*, 2006; Nomura *et al.*, 2004; Prins *et al.*, 2008). Most strategies involve the expression of inverted repeat (IR) or hairpin RNA (hpRNA) constructs sharing homology with the infecting viruses. In an engineered plant, hpRNAs that are transcribed from IR constructs are efficiently processed into siRNAs by dicer-like proteins

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(Dunoyer *et al.*, 2005; Fusaro *et al.*, 2006; Wesley *et al.*, 2001). The siRNAs are loaded into the RNA-induced silencing complex (RISC) containing Argonaute 1 (AGO1) (Baumberger and Baulcombe 2005). The siRNAs guide RISC to the viral genome by recognizing the complementary RNA sequence. The AGO1 slicer activity cleaves viral RNA, thereby rendering the plant resistant to the virus.

In contrast with plant viruses that normally encode multi-function RNA silencing suppressor proteins, viroids do not encode any proteins, and recent data have demonstrated that PSTVd itself does not function as an RNA silencing suppressor (Itaya *et al.*, 2007). PSTVd seems to have developed an alternative strategy to escape RNAi-mediated degradation. RISC appears to be mainly active in the cytoplasm and siRNAs produced in PSTVd-infected plants (srPSTVds; Itaya *et al.*, 2007; Machida *et al.*, 2007) are found in the cytoplasm, but not in the nucleus (Denti *et al.*, 2004). Thus, there is a possibility that RISC does not target PSTVd at its place of replication and accumulation. Systemic infection, however, requires the movement of the mature PSTVd through plasmodesmata into neighbouring cells and via the vascular system to distant organs. During movement, PSTVd must cross the cytoplasm and is exposed to the RNAi machinery. The failure of RISC-mediated targeting of mature viroid RNA has been postulated to be attributed to the highly ordered secondary structure of mature PSTVd (Itaya *et al.*, 2007; Wang *et al.*, 2004) and *Hop stunt viroid* (HSVd) RNA molecules (Gómez and Pallás, 2007). Alternatively, viroid RNA may be tightly associated with host proteins that shield it from the RNAi machinery. A recent study, however, has shown that the presence of high concentrations of double-stranded RNA (dsRNA) for PSTVd and two other viroids in the inocula confer sequence-specific inhibition of infection (Carbonell *et al.*, 2008), suggesting the susceptibility of viroid RNAs to RNAi.

In this study, we used an alternative approach to test whether PSTVd is susceptible to RNAi, an approach that may also be used directly to engineer viroid-resistant plants. In this approach, transgenic tomato lines that express a PSTVd IR construct were tested for resistance for PSTVd infection. We identified transgenic lines that accumulated high levels of PSTVd hpRNA-derived siRNAs (hp-siRNAs) and conferred effective resistance to PSTVd infection.

RESULTS

Transgenic tomato plant lines segregated into susceptible and resistant progeny for PSTVd infection

A segregating T3 population of the two transgenic tomato (*Solanum lycopersicum* cv. MoneyMaker) lines hpPSTVd-4/2 and hpPSTVd-11/8 (Wang *et al.*, 2004) was analysed for the presence of the PSTVd IR construct (hpPSTVd) by polymerase chain reaction

(PCR) (data not shown). Three cuttings (A, B, C) were generated from each of four transgene-containing seedlings (#11–#14). One cutting (A) was untreated and served as a control, and the other two cuttings (B and C) were each inoculated with 100 µg of total RNA from a PSTVd-infected *Nicotiana benthamiana* plant. At 12 weeks post-inoculation (wpi) symptom expression was examined. All inoculated cuttings of the four progeny of line hpPSTVd-4/2 exhibited disease symptoms (Fig. 1B,C) similar to those developed in PSTVd-infected MoneyMaker wild-type (MM) plants (Fig. 1, MM i). Stunting and leaf curling were the most obvious phenotypic alterations. In contrast with the hpPSTVd-4/2 cuttings, the inoculated cuttings of hpPSTVd-11/8 progeny plants were phenotypically indistinguishable from the non-inoculated cutting and from PSTVd-free MM plants (Fig. 1E,F, MM ni). Thus, the inoculated hpPSTVd-11/8 cuttings did not appear to be infected by PSTVd.

Importantly, and in contrast with the observations of Wang *et al.* (2004), none of the non-inoculated transgenic hpPSTVd lines developed apparent symptoms when compared with viroid-free wild-type plants (data not shown). It should be noted, however, that Wang *et al.* (2004) presented data on the T1 generation of hpPSTVd lines 3, 4 and 5, whereas we used individuals of the T3 generation of lines hpPSTVd 4 and 11 for the present study. The reasons for these different observations remain to be understood.

Symptom expression is correlated with PSTVd accumulation

To determine whether the observed symptoms in the inoculated transgenic lines were correlated with PSTVd infection, we performed Northern blot analysis with total RNA isolated from inoculated and non-inoculated plants using a full-length PSTVd cDNA as a probe. All samples from symptomatic inoculated hpPSTVd-4/2 cuttings showed clear hybridization signals. In contrast, no hybridization signals were detectable with RNA from inoculated hpPSTVd-11/8 cuttings. Representative examples of two cuttings of each line are shown in Fig. 2A. These results provide further evidence that progeny of the hpPSTVd-11/8 line are resistant to PSTVd infection. To further confirm PSTVd infection/resistance in the above cuttings, we conducted reverse transcriptase-polymerase chain reaction (RT-PCR) using a PSTVd-specific primer pair. As shown in Fig. 2C, a PCR product corresponding to the full-length PSTVd cDNA was amplified only from total RNA samples collected from inoculated MM plants and cuttings of line hpPSTVd-4/2, but not from inoculated cuttings of line hpPSTVd-11/8. It should be noted that inoculated MM plants were infected by PSTVd to 100% in all of our experiments, indicating that the PSTVd infection procedure was efficient. In addition, sequencing analysis of the RT-PCR products confirmed that the sequence of replicating PSTVd had not been altered. In

Fig. 1 Symptom expression in *Potato spindle tuber viroid* (PSTVd)-inoculated tomato plants carrying a PSTVd hairpin RNA construct at 12 weeks post-inoculation (wpi). PSTVd-infected (MM i) and PSTVd-free (MM ni) wild-type Moneymaker plants were used as controls. Representative examples of two cuttings of one progeny from the lines hpPSTVd-4/2 and hpPSTVd-11/8 are shown. The PSTVd-inoculated hpPSTVd-4/2 plants (B, C) develop disease symptoms comparable with those of MM i plants. In contrast, all plants from line hpPSTVd-11/8 (E, F) appear to be symptomless. The plants on the left (A, D) are non-PSTVd-inoculated cuttings of the two lines.

summary, disease symptom expression was clearly correlated with PSTVd infection, with symptomless plants showing no viroid accumulation.

PSTVd resistance is correlated with high-level hpPSTVd siRNA accumulation

To examine whether PSTVd resistance in transgenic plants was mediated by RNAi, we analysed the accumulation of siRNAs corresponding to PSTVd sequences in these plants prior to and after PSTVd inoculation. Prior to PSTVd inoculation, no PSTVd-specific small RNAs were detectable in non-inoculated MM plants (Fig. 3, lane 5), but hp-siRNAs were present in the cuttings of the two transgenic lines. Interestingly, the hp-siRNA concentration varied between the two different lines. As exemplified for two individuals of each line, a relatively low concentration of 21- and 24-nucleotide siRNAs was found in progeny plants of line hpPSTVd-4/2 (Fig. 3, lanes 3 and 4). In contrast, a high level of accumulation of hp-siRNAs was detectable in progeny plants of line hpPSTVd-11/8 (Fig. 3, lanes 1 and 2). From the analysis of all of our

experiments, it was calculated that hp-siRNA accumulation in non-inoculated hpPSTVd-11/8 progeny plants was approximately 2.7-fold higher than that in non-inoculated hpPSTVd-4/2 progeny plants. Thus, there seemed to be a correlation between high-level accumulation of hp-siRNA and PSTVd resistance.

At 12 wpi, the accumulation of PSTVd-specific siRNAs was re-examined in the cuttings. Compared with the concentrations prior to PSTVd inoculation, the siRNA levels had increased significantly in cuttings of line hpPSTVd-4/2 (compare lanes 3 and 4 with lanes 8 and 9 in Fig. 3). It is probable that this high level was a result of PSTVd infection giving rise to srPSTVds. The assumption was in accordance with the detection of additional strong hybridization signals at the top of the gel in the samples of the inoculated MM plant and the cuttings of line hpPSTVd-4/2 (Fig. 3, lanes 8–10; arrow). The hybridizing high-molecular-weight (HMW) RNA could be assigned to mature PSTVd molecules derived from autonomous viroid RNA/RNA replication. In the cuttings from the hpPSTVd-11/8 line, the siRNA levels were not significantly different before and after PSTVd inoculation. The absence of HMW RNA hybridization signals indicated that only

Fig. 2 Northern blot and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *Potato spindle tuber viroid* (PSTVd)-inoculated tomato plants carrying a PSTVd hairpin RNA construct at 12 weeks post-inoculation (wpi). (A) Northern blot analysis of the plants shown in Fig. 1. Lane 1, PSTVd-inoculated Moneymaker wild-type plant; lane 2, non-PSTVd-inoculated cutting A of progeny 12 of line hpPSTVd-4/2; lanes 3 and 4, PSTVd-inoculated cuttings B and C of progeny 12 of line hpPSTVd-4/2; lane 5, non-PSTVd-inoculated cutting A of progeny 12 of line hpPSTVd-11/8; lanes 6 and 7, PSTVd-inoculated cuttings B and C of progeny 12 of line hpPSTVd-11/8. (B) Image of ethidium bromide-stained and membrane-bound RNA for use as a RNA loading control. (C) RT-PCR analysis of the plants shown in Fig. 1 using the PSTV-Nb-F/R primer pair to produce the full-length PSTVd cDNA. Lane 1, RT-PCR products from a PSTVd-inoculated Moneymaker wild-type plant; lane 2, products from a non-PSTVd-inoculated Moneymaker wild-type plant; lane 3, RT-PCR products from a sample lacking RNA (negative control); lanes 4 and 5, products from the PSTVd-inoculated cuttings B and C of progeny 12 of line hpPSTVd-4/2; lanes 6 and 7, PSTVd-inoculated cuttings B and C of progeny 12 of line hpPSTVd-11/8. M, DNA marker (GeneRuler™ 1 kb-DNA-Ladder-Mix, Fermentas, St. Leon-Roth, Germany).

Fig. 3 Analysis of hairpin RNA-derived small interfering RNA (hp-siRNA) accumulation in tomato plants carrying a *Potato spindle tuber viroid* (PSTVd) hpRNA construct prior to and after PSTVd inoculation. RNA samples isolated from non-PSTVd-inoculated [0 weeks post-inoculation (wpi)] and from inoculated (12 wpi) tomato cuttings were hybridized with a full-length PSTVd cDNA as probe. Lanes 1 and 2, cuttings B of progeny plants 11 and 12 of line hpPSTVd-11/8 prior to inoculation; lanes 3 and 4, cuttings B of progeny plants 11 and 12 of line hpPSTVd-4/2 prior to inoculation; lane 5, non-inoculated Moneymaker wild-type plant; lanes 6 and 7, inoculated cuttings B of progeny plants 11 and 12 of line hpPSTVd-11/8; lanes 8 and 9, inoculated cuttings B of progeny plants 11 and 12 of line hpPSTVd-4/2; lane 10, inoculated Moneymaker wild-type plant. Note that hp-siRNAs with sizes between 21 and 24 nucleotides are detectable in all samples. An additional high-molecular-weight (HMW) hybridization signal was found in the RNA samples from the two PSTVd-inoculated hpPSTVd-4/2 and the PSTVd-inoculated Moneymaker wild-type plant (arrow). The percentage values shown below the blot image indicate the relative hp-siRNA amounts among the four samples. These values were calculated on the basis of the intensity of the hp-siRNA signals and that of the larger cross-hybridizing band.

cuttings from line hpPSTVd-11/8 were PSTVd resistant. In summary, the data indicate that plants with relatively high levels of hp-siRNA accumulation show PSTVd resistance, whereas those with low levels of hp-siRNAs are susceptible to PSTVd infection.

Screening of additional PSTVd resistant lines

In order to identify additional lines displaying PSTVd resistance, progeny plants of transgenic tomato were examined by Southern blot analysis. Genomic DNA was isolated from seedlings (indicated by 'a') of the three available T3 lines: hpPSTVd-4/1,

Fig. 4 Southern blot analysis of tomato plants carrying a *Potato spindle tuber viroid* (PSTVd) hairpin RNA construct. (A) Schematic presentation of the T-DNA containing the PSTVd hairpin RNA construct. (B) Southern blot analysis of genomic DNA isolated from a wild-type Moneymaker plant (MM, lane 2), seedling 4/1a of line hpPSTVd-4/1 (lane 3), seedling 4/2a of line hpPSTVd-4/2 (lane 4) and seedling 11/8a of line hpPSTVd-11/8 (lane 5). The DNA was digested with *Xba*I and the membrane was hybridized with a T-DNA right border probe (A). (C) Southern blot analysis of the same DNA samples as in (B) but digested with *Sca*I. The membrane was hybridized with a T-DNA left border probe (A). BL, T-DNA left border; pAnos, polyadenylation signal sequence of the nopaline synthase gene; NPT II, neomycin phosphotransferase gene; Pnos, nopaline synthase gene promoter; P35S, *Cauliflower mosaic virus* 35S promoter; vertical lines in the left PSTVd cDNA unit indicate the deletions (see also Fig. 7); pAocs, polyadenylation signal sequence of the octopine synthase gene; BR, T-DNA right border; M, DNA marker (GeneRuler™ 1 kb-DNA-Ladder-Mix, Fermentas; the 2, 5 and 10-kb fragments are indicated).

hpPSTVd-4/2 and hpPSTVd-11/8. The DNA was cut with *Sca*I and *Xba*I to enable separate analysis of the left and right border integration sites of the T-DNA (Fig. 4A). The three seedlings examined showed unique left (Fig. 4B) and right (Fig. 4C) border-specific hybridization patterns of these three lines. The hpPSTVd-4/1a seedling carried a single transgene locus, whereas the hpPSTVd-11/8a seedling harboured two and the hpPSTVd-4/2a seedling at least three transgene copies. Four and two fragments hybridized with the right (Fig. 4B, lane 4) and left border-specific probes (Fig. 4C, lane 4), respectively. This indicated transgene rearrangements in this line.

RNA from these three seedlings was extracted for siRNA accumulation analysis. Evaluation of the hp-siRNA concentration of seedlings hpPSTVd-11/8a and hpPSTVd-4/2a was roughly in

agreement with our previous findings in other seedlings of these lines (Fig. 5A, lane 2 and 3). The hp-siRNA level was approximately 2.1- and 1.3-fold higher in hpPSTVd-11/8a than in hpPSTVd-4/2a and hpPSTVd-4/1a seedlings, respectively. In the hpPSTVd-4/1a seedling, hp-siRNA accumulation was approximately 1.6-fold higher than in hpPSTVd-4/2a, demonstrating that the hp-siRNA level did not correlate with the transgene copy number.

After leaf samples had been taken for DNA and RNA extraction, the three plants were inoculated with PSTVd. At 12 wpi, only the hpPSTVd-4/2a seedling had developed severe symptoms (Fig. 5B). The hpPSTVd-11/8a and hpPSTVd-4/1a seedlings did not show any phenotypic alterations, indicating that they were PSTVd resistant. To confirm that seedlings hpPSTVd-11/8a and hpPSTVd-4/1a were indeed PSTVd free, RNA samples from all

Fig. 5 Disease symptoms of tomato plants carrying a *Potato spindle tuber viroid* (PSTVd) hairpin RNA (hpRNA) construct, and small interfering (siRNA) analysis of these plants prior to PSTVd inoculation. (A) siRNA detection: RNA samples were isolated from non-inoculated seedlings 4/1a, 4/2a and 11/8a (lanes 1–3) that were used for the Southern blot analysis shown in Fig. 4. The RNAs were hybridized with a full-length PSTVd cDNA as probe. hp-siRNAs with sizes between 21 and 24 nucleotides were detectable in all samples. The percentage values and evaluation of the relative hp-siRNA levels were performed as described in Fig. 3. (B) Disease symptoms [12 weeks post-inoculation (wpi)] of PSTVd-inoculated cuttings of seedlings 11 and 12 of the hpPSTVd-4/1, hpPSTVd-4/2 and hpPSTVd-11/8 lines (cuttings B).

three plants were applied to RT-PCR analysis, as described above. The PSTVd-specific primer pair amplified the full-length PSTVd cDNA with RNA samples from an infected Moneymaker wild-type and symptom-expressing hpPSTVd-4/2a plants (Fig. 6, lanes 2 and 4). In contrast, no PCR products were amplified with RNA of seedlings hpPSTVd-4/1a and hpPSTVd-11/8a (Fig. 6, lanes 3 and 5). To confirm the above data, eight plants of each genotype were analysed. With respect to hp-siRNA levels and PSTVd susceptibility/resistance, the same results were obtained (data not shown). In summary, of the three different transgenic tomato lines comprising 24 individual plants, two lines were resistant to PSTVd. In the resistant plants, the levels of hp-siRNA accumulation were higher than those in susceptible plants.

Fig. 6 Reverse transcriptase-polymerase chain reaction (RT-PCR) detection of *Potato spindle tuber viroid* (PSTVd). Total RNA was isolated from a PSTVd-infected Moneymaker wild-type plant (MM 1i) (lane 2) and from PSTVd-inoculated seedlings 4/1a, 4/2a and 11/8a (lanes 3–5) at 12 weeks post-inoculation (wpi). RNA was applied to RT-PCR amplification using a PSTVd-specific primer pair. With these primers, the full-length PSTVd cDNA (arrowhead) was only detected from PSTVd-infected wild-type and hpPSTVd-4/2a plants. M, DNA marker (GeneRuler™ 1 kb-DNA-Ladder-Mix, Fermentas)

Temperature sensitivity of siRNA accumulation

Transgene-produced siRNA levels have been demonstrated to be positively correlated with increasing temperature in *Nicotiana* species (Kalantidis *et al.*, 2002; Szittyta *et al.*, 2003). Thus, we examined whether, in the hpPSTVd tomato lines, hp-siRNA accumulation was temperature dependent. Cuttings of one seedling from lines hpPSTVd-4/1, hpPSTVd-4/2 and hpPSTVd-11/8 were grown under identical light conditions, but at 21 and 31 °C. After the plants had been maintained for 6 weeks under these conditions in growth chambers, RNA was extracted and applied to small RNA analysis. Instead of increased hp-siRNA concentrations, we observed slightly reduced levels at 31 °C in lines hpPSTVd-4/1 and hpPSTVd-4/2. In the seedling of line hpPSTVd-11/8, the hp-siRNA level was approximately 50% lower at the high temperature than at 21 °C (data not shown).

Profiling of PSTVd-specific siRNA in transgenic plants

PSTVd infection activates the plant RNA-mediated defence mechanism and results in the accumulation of high concentrations of

Fig. 7 Genomic map locations of hairpin RNA-derived small interfering RNAs (hp-siRNAs) from hpPSTVd-11/8 transgenic plants. Blue and red lines represent siRNAs from (+)- and (-)-strands, respectively. Broken lines delineate the boundaries among the five structural domains: left terminal, pathogenicity, central, variable and right terminal (Keese and Symons, 1985). The deleted sense *Potato spindle tuber viroid* (PSTVd) sequences in the hpPSTVd construct are highlighted in grey.

srPSTVds. Nevertheless, PSTVd readily establishes systemic infection in a host, indicating that srPSTVds cannot effectively target the viroid genome for degradation. Our observation that siRNAs originating from a PSTVd IR construct could target and silence the PSTVd genome raised the question of whether hp-siRNAs differ from srPSTVds. In an attempt to answer this question, we cloned and sequenced some hp-siRNAs (Fig. 7). It should be noted that hpRNA lacks 19 nucleotides (Fig. 7, $\Delta 356-15$) in the left terminal domain and 12 nucleotides (Fig. 7, $\Delta 157-168$) in the right terminal domain of the sense strand. Therefore, the PSTVd IR transgene should not produce hp-siRNAs corresponding to these regions. Among the small RNA sequences obtained, 75% of sequences were of plus strands and 25% were of minus strands. Overall, the majority of plus-strand small RNAs were similar between hp-siRNAs and srPSTVds, deriving from the variable or right terminal regions (Itaya *et al.*, 2007; Machida *et al.*, 2007). The distribution of the minus-strand hp-siRNAs was also similar to the srPSTVds reported by Machida *et al.* (2007).

DISCUSSION

In this study, we examined the effectiveness of hpRNA-derived siRNAs in mediating resistance to PSTVd infection using transgenic tomato expressing a PSTVd IR construct. Compared with the simultaneous introduction of dsPSTVd RNA with PSTVd inoculum in plants (Carbonell *et al.*, 2008) and dsPSTVd or siRNA with PSTVd inoculum in protoplasts (Itaya *et al.*, 2007), the use of hpRNA transgenic plants in this study allowed for the testing of the ability of PSTVd to infect plants with pre-existing PSTVd-specific siRNAs. This mimics the strategy likely to be used for the generation of viroid-resistant transgenic plants. Indeed, screening of three independent transgenic lines led to the identification of two lines that conferred high-level resistance (lines hpPSTVd-4/

1a and hpPSTVd-11/8a) and another line that displayed susceptibility (hpPSTVd-4/2a) to PSTVd infection. We showed that this resistance appeared to correlate with the level of hp-siRNAs. It is, however, intriguing that plants exhibiting PSTVd resistance contained only 1.6-fold more hp-siRNAs than susceptible plants. Although this may suggest a requirement for a threshold hp-siRNA level for effective viroid resistance, it remains to be investigated further whether it is solely the higher siRNA level, or this level in addition to other factors, that accounts for the resistance. Nevertheless, our result strongly indicates that the expression of a PSTVd IR construct can lead to effective PSTVd resistance in tomato.

Exogenously supplied siRNAs or dsPSTVd RNAs failed to silence replicating PSTVd or green fluorescent protein (GFP):PSTVd fusion constructs in *N. benthamiana* protoplasts (Itaya *et al.*, 2007). However, although the structured PSTVd RNA appeared to be resistant to RNA silencing (Itaya *et al.*, 2007), PSTVd may assume diverse conformations *in vivo*, some of which could be susceptible to silencing. The presence of sufficient siRNAs at the appropriate stage of the PSTVd infection cycle, and in the right subcellular compartment(s), may be important for efficient silencing. Furthermore, as suggested here by our result, and shown by Carbonell *et al.* (2008), perhaps high levels of siRNAs are necessary to overcome the secondary structure-based viroid resistance to silencing.

It should also be noted that, rather than *N. benthamiana*, tomato plants were used in this study. Tomato has been reported occasionally to recover from PSTVd infection. Interestingly, the accumulation of siRNAs preceded recovery, indicating a correlation between recovery and RNA silencing (Sano and Matsuura, 2004). Unlike *N. benthamiana*, tomato contains a functional RNA-directed RNA polymerase 1 (RDR1) (Yang *et al.*, 2004). RDR1 activity is elevated in virus-infected plants (Dorssers *et al.*,

1984; Zabel *et al.*, 1974), and it is assumed to be associated with the control of virus accumulation in tobacco and *Arabidopsis thaliana* (Xie *et al.*, 2001; Yu *et al.*, 2003). At least in tomato, RDR1 is also induced on PSTVd infection (Schiebel *et al.*, 1993, 1998). Thus, it is reasonable to speculate that RDR1 is involved in the control of viroid RNA accumulation, probably by copying viroid RNA to produce dsRNA (Wassenegger and Krczal, 2006). However, it is not clear whether hp-siRNAs would directly contribute to such a mechanism. The fact that recovery from PSTVd infection occurs in non-transgenic plants suggests that RDR1-mediated control of viroid RNA accumulation, if it exists, functions independently of hp-siRNAs.

The hpPSTVd lines analysed here did not show elevated hp-siRNA accumulation when grown at 31 °C. RNA samples were taken after 2, 4 and 6 weeks, but at none of these time points were hp-siRNA levels increased at 31 °C (data not shown). This is in contrast with reports on the temperature-dependent accumulation of transgene-derived siRNAs in *N. tabacum* and *N. benthamiana* (Kalantidis *et al.*, 2002; Szittyá *et al.*, 2003). Additional tomato plants exhibiting transgene-mediated gene silencing must be characterized to determine whether transgene-produced siRNA accumulation is indeed temperature independent in this plant species.

It was unexpected that the hp-siRNAs showed similar distribution patterns to srPSTVds. Several possible scenarios could account for this similarity. First, instead of forming the predicted hpRNA, the arms of the PSTVd IR transcripts may form secondary structure(s) similar to that of replicating PSTVd RNA. Conversely, srPSTVds may not originate from dicer processing of the stem-loop structures formed by mature PSTVd RNA, but may be mainly secondary siRNAs derived from RDR6-synthesized dsRNAs. Consistent with this, high accumulation of HSVd-specific siRNAs in transgenic *N. benthamiana* is dependent on RDR6 activity (Gomez *et al.*, 2008), suggesting that the major fraction of accumulating viroid siRNAs is represented by secondary siRNAs generated via RDR6. It has been suggested previously that the highly structured (+)- and (–)-strands of PSTVd RNA might be differentially amplified by plant RDRs (Vogt *et al.*, 2004). Thus, RDR6 may only produce dsRNA corresponding to parts of PSTVd RNAs, explaining why accumulating hp-siRNAs and srPSTVds map to similar regions.

Another possible scenario is that some small RNA populations may not be cloned as efficiently as other small RNA populations, causing biased representation of small RNA distributions. We did not recover small RNA sequences of 24–25 nucleotides in length, despite the detection of these hp-siRNA species by Northern blotting. The same phenomenon was reported by Machida *et al.* (2007) for srPSTVds in PSTVd-infected tomato plants. It is possible that long hp-siRNAs or srPSTVds carry chemical properties that make cloning difficult. It is important to emphasize that sequencing is not saturated in all of these studies, which may also

partially account for these 'unexpected' observations. Our observations underscore the importance of continuing studies with deep sequencing and functional analyses to further understand the significance of viroid small RNAs in infection and pathogenicity.

Finally, the uneven distribution of PSTVd siRNAs along the PSTVd genome could be accounted for by the inaccuracy of dicer-like protein(s) (Rajagopalan *et al.*, 2006). Only dicer products with sizes between 21 and 24 nucleotides would be loaded onto an AGO protein and would therefore preferentially accumulate. In addition, the preference of AGO proteins for specific 5' nucleotides during small RNA loading has been reported (Mi *et al.* 2008). Conceivably, only small RNAs with 5' terminal nucleotides that are preferentially loaded onto nuclear AGO proteins accumulate efficiently. This could result in the differential accumulation of siRNAs from different regions in the PSTVd genome.

In conclusion, the inhibition of PSTVd infection in transgenic tomato expressing a PSTVd IR construct, reported here, and similar inhibition in non-transgenic plants via the use of dsRNAs co-delivered mechanically or co-agroinoculated, as observed by Carbonell *et al.* (2008), suggest that RNAi is active in the underlying mechanism for the observed viroid resistance. Interestingly, Carbonell *et al.* (2008) showed that co-inoculation of dsRNA also caused sequence-specific inhibition of infection by *Citrus exocortis viroid* (another member of *Pospiviroidae*) and *Chrysanthemum chlorotic mottle viroid* (a member of *Avsunviroidae*). Thus, RNAi may be an effective approach for engineering crop resistance to a broad range of viroids. We are currently investigating whether artificial microRNA (amiRNA) constructs, which have been successfully employed to generate virus resistance in plants (Niu *et al.*, 2006), can be used to target the conserved region of *Pospiviroidae* genomes, giving effective resistance to the viroids. Wang *et al.* (2004) observed PSTVd hp-siRNA-mediated symptom development in tomato. The amiRNA strategy would minimize such target-off effects. amiRNAs may be screened for those which would confer viroid resistance, but would not interfere with normal plant gene regulation. In addition and in contrast with siRNA accumulation, amiRNA biosynthesis has been demonstrated to be temperature independent. Although we did not find increased PSTVd hp-siRNA levels at 31 °C, when compared with hp-siRNA accumulation at 21 °C, differential hp-siRNA accumulation was detectable at other temperature gradients, e.g. between 15 and 24 °C.

EXPERIMENTAL PROCEDURES

Plant material, growth conditions and viroid sources

Tomato (*S. lycopersicon* cv. Moneymaker) and *N. benthamiana* plants were grown in a glasshouse with a 16-h light (24 °C) and 8-h dark (24 °C) cycle. The plants were watered daily with a weekly addition of commercial plant nutrients as instructed by

the manufacturer (Compo Blaukorn® Entec®, Compo GmbH & Co. KG, Münster, Germany). Seeds of the transgenic tomato plants carrying the hpPSTVd constructs (lines hpPSTVd-4/1, hpPSTVd-4/2 and hpPSTVd-11/8) were obtained from Ming-Bo Wang. The generation of these plant lines has been described previously (Wang *et al.*, 2004). Transgenic tomato plants were grown in Versatile Environmental Test Chambers MLR-351 (Sanyo Electric Co., Osaka, Japan) for maintenance at 21 or 31 °C.

Plant inoculation

Inoculation of tomato plants was performed basically as described by Wassenegger *et al.* (1996). The inoculum contained 100 µg of total RNA isolated from a PSTVd-Nb (GENBANK Accession No. AJ634596)-infected *N. benthamiana* plant per 100 µL of buffer. PSTVd-infected *N. benthamiana* plants were produced as described by Qi *et al.* (2004).

Extraction of nucleic acids from plants

Plant DNA for PCR analysis was extracted using the Qiagen DNeasy Plant Mini-Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For Southern blot analysis, plant DNA was isolated according to Dellaporta *et al.* (1983). Two procedures were applied for total RNA extraction. RNA that was subsequently used to inoculate plants was isolated from 1 g of leaf material according to Logemann *et al.* (1987). For Northern blot analysis and siRNA detection, total RNA was extracted from leaf material using Tri-Reagent (Sigma-Aldrich, Steinheim, Germany) following the manufacturer's instructions. The RNA was dissolved in 50% formamide.

Southern blot analysis

Ten micrograms of tomato genomic DNA were digested with restriction enzymes *ScaI* and *XbaI* (Roche, Manheim, Germany) for 16 h. The digested DNA was separated on 1% Tris-acetate and EDTA agarose gels at 25 V for 20 h. Before the DNA was transferred onto positively charged nylon membranes (BioBond-Plus Nylon membrane, Sigma-Aldrich), the agarose gels were soaked once in 0.25 M HCl for 10 min and twice in 0.4 M NaOH, 1 M NaCl for 20 min. Capillary transfer was performed overnight with 0.4 M NaOH, 1 M NaCl. Hybridization was performed according to Amasino (1986) using random primed [α -³²P]dCTP-labelled DNA (Random Primed DNA Labelling Kit, Roche) as probes. The probes were produced by cutting the binary vector pMBW380 (Wang *et al.*, 2004) with *NotI/ScaI* and *SacI/XbaI*. The *NotI/ScaI* digestion generated a 720-bp fragment that was used to specifically detect the 'left border/plant DNA' junction. The 800-bp *SacI/XbaI* fragment was used to detect the 'right border/plant DNA' junction. Hybridizing DNA fragments were visualized as described below in the section on 'Imaging and analysis of hybridization signals'.

Northern blot analysis and siRNA detection

Northern blot analysis was performed as described previously (Vogt *et al.*, 2004) with the following modifications. Five micrograms of total RNA per lane were loaded and separated on a 1.2% formaldehyde-containing agarose gel (13 cm × 13 cm). RNA was transferred onto positively charged nylon membranes (BioBond-Plus Nylon membrane, Sigma-Aldrich). The transfer buffer consisted of 10 × standard saline citrate (SSC), and hybridization was performed at 64 °C in a formamide-free buffer system [250 mM sodium phosphate, 7% sodium dodecylsulphate (SDS), 2.5 mM ethylenediaminetetraacetic acid (EDTA), pH 7.2]. The membranes were washed twice at 64 °C with buffer 1 (1 × SSC, 0.1% SDS, 30 min) and buffer 2 (0.5 × SSC, 0.1% SDS, 30 min). Hybridizing signals were visualized by autoradiography after exposure at –80 °C for 5 h using Scientific Imaging Films (Kodak BioMax MS Film, Sigma-Aldrich).

siRNA detection was conducted according to the protocol described by Himber *et al.* (2003). A total of 0.5 µg of RNA was separated utilizing the Invitrogen Xcell sure lock system (Invitrogen, Karlsruhe, Germany) with ready-to-use 20% Tris base, boric acid and EDTA gels (Anamed, Groß-Bieberau, Germany). The transfer of RNA onto positively charged nylon membranes (BioBond-Plus Nylon membrane, Sigma-Aldrich) was performed using the Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories GmbH, Munich, Germany). RNA separation and blotting were conducted according to the manufacturer's instructions. For Northern blotting and siRNA detection, a random primed [α -³²P]dCTP-labelled (Random Primed DNA Labelling Kit, Roche) full-length PSTVd cDNA was used as a probe.

Imaging and analysis of hybridization signals

Images of Southern and siRNA detection blots were either produced using scientific imaging films (Kodak Biomax MS, Sigma-Aldrich) or the Molecular Imager system, Pharos FX Plus (Bio-Rad Laboratories GmbH). The phosphor screens were scanned using the imaging and analysing software Quantity One® Ver. 4.6.3 (Bio-Rad Laboratories GmbH). Images were taken after 5 h and 19 h (siRNA detection) or 48 h (Southern blots) of exposure time for both films and imager screens.

To enable the evaluation of relative hp-siRNA accumulation, the levels of cross-hybridization bands were used as an internal control. The PSTVd probe cross-hybridized with endogenous tomato sequence(s), resulting in a distinct hybridizing band (see the strong middle band in Fig. 3, lane 5). The signal strength of these bands was determined using Quantity One® Ver. 4.6.3 (Bio-Rad Laboratories GmbH) analysing software, and these values were used as a measure of RNA concentration. This approach provided solid data and was favoured over the comparison of ribosomal RNA concentrations or photometric evaluation of total RNA levels. The most intense signal strengths

of the cross-hybridizing and hp-siRNA bands were each set to 100%. Calculation of the absolute hp-siRNA level per lane was achieved by adjusting the RNA loading of each sample to 100%.

PCR and RT-PCR analysis of progeny plants

Progeny plants were screened for the presence of the transgene construct by PCR using 50 ng of genomic DNA and the primer pair MBW-Spacer-PCR Fwd 2 (5'-CATTGATCTTACATTGGATTG-3', 10 µM)/PA-OCT-PCR (5'-CATGCGATCATAGGCGTCTC-3', 10 µM). In plants containing the transgene construct, a 425-bp product was amplified with Taq DNA Polymerase (5 Prime GmbH, Hamburg, Germany): 94 °C, 5 min; (94 °C, 30 s; 50 °C, 30 s; 72 °C, 1 min) × 35; 72 °C, 4 min.

In addition to Northern blot analysis, RT-PCR was applied to screen PSTVd-inoculated plants. From 200 ng of total RNA, the full-length PSTVd cDNA was amplified using a SuperScript™ III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen) and with the primer pair PSTV-Nb-R (5'-GGATCCCTGAAGCGCTCCTCC-3', 10 µM)/PSTV-Nb-F (5'-GGATCCCCGGGGAACCTGGA-3', 10 µM). The reaction conditions were as follows: 60 °C, 30 min; 94 °C, 2 min; (94 °C, 15 s; 55 °C, 30 s; 68 °C, 45 s) × 40; 68 °C, 5 min.

Sequencing of hp-siRNAs

The cloning and sequencing of hp-siRNAs were performed essentially as described by Itaya *et al.* (2007). One gram of leaf tissue from hpPSTVd-11/8 transgenic plants was used for each cloning/sequencing experiment. Three biological replicates were performed and all sequences obtained were combined for presentation.

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