Effects of viral silencing suppressors on tobacco ringspot virus infection in two Nicotiana species

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This study investigated the effects of silencing suppressors derived from six different viruses (P1, P19, P25, HcPro, AC2 and 2b), expressed in transgenic Nicotiana tabacum and Nicotiana benthamiana plants, on the infection pattern of tobacco ringspot virus (TRSV) potato calico strain. In N. benthamiana, this virus produced an initial infection with severe systemic symptoms, but the infection was strongly reduced within a few weeks as the plant recovered from the infection. P25 and HcPro silencing suppressors effectively prevented recovery in this host, allowing continuous accumulation of the viral RNA as well as of the virus-specific small interfering RNAs, in the systemically infected leaves. In the P1-, P19-, AC2- or 2b-expressing transgenic N. benthamiana, the recovery was not complete. Susceptibility of N. tabacum to this virus was temperature sensitive. At lower temperatures, up to 25 °C, the plants became systemically infected, but at higher temperatures, the infections were limited to the inoculated leaves. In these preventative conditions, all silencing suppressor transgenes (except P25, which was expressed at very low levels) allowed the establishment of systemic infections. Very strong and consistent systemic infections were observed in HcPro- and AC2-expressing plants.

INTRODUCTION

RNA silencing is a complex mechanism mediating different types of sequence-specific gene regulation and defence reactions, including downregulation of gene expression, induction and maintenance of methylation of homologous DNA sequences, and surveillance and defence against invading genetic elements such as transposons, transgenes and viral pathogens (Lehto & Siddiqui, 2005). The mechanism is activated by dsRNAs, which are recognized and cleaved by the Dicer (or Dicer-like) enzyme into small fragments (21–26 nt), which subsequently associate with the RNA-induced silencing complex and direct either the degradation or translational arrest of their homologous target RNA sequences (Matzke et al., 2004; Deleris et al., 2006; Zhang et al., 2006). Overall, the silencing-related pathways are very complex, and at least in plants and in some animals they include local and systemic spread of the silencing signal in the organism and amplification of the silencing process after its initiation in new cells (reviewed by Voinnet, 2005b; Buchon & Vaury, 2006).

In plants, the silencing pathways are effectively induced by dsRNAs produced by replicating viral genomes or by secondary structures of viral transcripts. Silencing reactions provide an efficient antiviral defence system (Ratcliff et al., 1997; Voinnet et al., 1999; Waterhouse et al., 2001; Lecellier & Voinnet, 2004; Roth et al., 2004; Lehto & Siddiqui, 2005; Qu & Morris, 2005; Voinnet, 2005a), and many plant viruses produce specific silencing suppressor proteins to overcome this defence. The essential role of silencing-mediated defences and the virus-encoded counter defences is indicated by the fact that many of the viral silencing suppressors have been identified previously as pathogenicity factors essential for viral infectivity (Brigneti et al., 1998; Roth et al., 2004; Voinnet, 2005a). Silencing may still remain a limiting factor for virus accumulation and pathogenicity, as some strong suppressor proteins, when expressed in transgenic plants, may strongly enhance the accumulation and symptoms of other viruses in these plants (Pruss et al., 2004). Enhanced silencing suppression is also the cause of synergistic effects of mixed viral...
infections (Pruss et al., 1997; Kasschau & Carrington, 1998; González-Jara et al., 2005).

A very pronounced example of the efficient plant defence mechanism is the exclusion of most of the viral pathogens from the meristematic tissues. This defence appears to be mediated by an RNA silencing-based surveillance system, which also excludes the entry of long-distance silencing signals to the meristem (Foster et al., 2002; Schwach et al., 2005). When the silencing process is activated in the developing leaf tissues, it may lead to exclusion or strong reduction of virus accumulation in these tissues, i.e. to ‘recovery’ of newly grown tissues from the disease. The recovered tissues are resistant to secondary infections of viruses carrying homologous sequences, thus proving that recovered tissues are resistant to secondary infections of these viruses, i.e. to developing leaf tissues, it may lead to exclusion or strong reduction of virus accumulation in these tissues, i.e. to ‘recovery’ of newly grown tissues from the disease.

Here, we analysed how different virus-derived silencing suppressors, expressed in transgenic Nicotiana tabacum Xanthi and Nicotiana benthamiana lines, affected accumulation, systemic spread, symptom severity and possible recovery in a nepovirus infection. As the experimental system, we used the potato calico strain of tobacco ringspot virus (TRSV), which in N. benthamiana induces very clear initial ringspot symptoms, with obvious later recovery.

METHODS

Plant material. N. tabacum Xanthi NN and N. benthamiana plants were transformed using Agrobacterium tumefaciens carrying the pBin61 binary plasmid alone, or containing the viral silencing suppressor gene P1 from rice yellow mosaic virus (RYMV), P19 from tomato bushy stunt virus, P25 from potato virus X, HcPro from cucumber mosaic virus (CMV), strain Kin. The transgene-positive lines were propagated to the R2 generation. From this progeny, two homozygote lines were initially characterized (Siddiqui et al., 2008). TRSV inoculations were carried out with two representative lines, with similar results obtained for both lines, and the final results are presented for one selected line.

TRSV inoculum, inoculation methods and sample collection. TRSV, potato calico strain, was obtained from the DSMZ. The virus was propagated in N. benthamiana plants and purified from systemically infected leaves using a nepovirus purification procedure (Frison & Stace-Smith, 1992). The 2′-terminal sequences of the purified viral genomic RNAs were copied into cDNA using an oligo(dT) anchored primer and a 5′3′ RACE kit, 2nd Generation (Roche), according to the manufacturer’s instructions. Using the same kit, the cDNA was poly(A)-tailed at its 3′ end and amplified by PCR with the oligo(dT) anchored primers. The 458 nt PCR product was cloned and sequenced using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and an ABI PRISM 3130xl Genetic Analyser (Applied Biosystems). With the exception of 60 3′-terminal nucleotides, the sequence had 77% identity with the 3′-terminal sequences of TRSV type isolate RNAs 1 and 2.

Plants were maintained under standard greenhouse conditions (25 °C, 16 h photoperiod at 100–200 μmol m⁻² s⁻¹). The two uppermost leaves of 3-week-old plants (transgenic and non-transformed N. benthamiana and N. tabacum Xanthi plants) were mechanically inoculated with TRSV, obtained by grinding TRSV systemically infected leaves in 10 mM phosphate buffer (pH 7.0). The inoculated N. tabacum non-transformed plants were grown at 18, 25, 26–29 or 33 °C. The inoculated N. tabacum transgenic plants were grown at 25 or 26–29 °C, and the inoculated N. benthamiana plants were grown at 25 °C. The photoperiod was always 16 h at 100–200 μmol m⁻² s⁻¹. Samples were collected for Northern blot analysis from the uppermost, fully grown systemic leaves at 7–10 and 35–40 days post-inoculation (p.i.), as indicated in Results. Samples were also collected from inoculated leaves at 7–10 days p.i. from the N. tabacum plants. One sample was analysed from each plant.

RNA extraction, Northern blot and siRNA analyses. Total RNA was extracted from the collected samples as described previously (Siddiqui et al., 2007). Northern blot detection of TRSV-specific RNAs was carried out using a digoxigenin-labelled probe, synthesized by PCR from the above-mentioned cDNA clone, according to the manufacturer’s instructions (Roche). Analysis of TRSV-specific small interfering RNAs (siRNAs) was carried out according to Sarmiento et al. (2006) using 30 μg total RNA from N. benthamiana. The radioactive probe was a 32P-labelled in vitro transcript corresponding to the above-mentioned cDNA clone of the viral RNAs. Radioactive signals were detected with a Personal Molecular Imager FX system (Bio-Rad).

RESULTS

TRSV infection in N. benthamiana plants expressing various silencing suppressors

Non-transformed wild-type (wt) as well as all of the transgenic N. benthamiana plants exhibited typical TRSV symptoms within 1 week of inoculation (Fig. 1a and data not shown). Northern blot analysis from systemically infected leaves at 10 days p.i. showed that TRSV accumulated effectively in all plants, and the accumulation was clearly enhanced in the transgenic plants expressing the P19, P25 and HcPro genes (Fig. 2).

Non-transformed plants and plants transformed with the empty pBin61 vector started to recover from infection at 3–4 weeks p.i. After 5 weeks p.i., the newly emerging leaves in these plants looked healthy and contained hardly any TRSV RNA (Figs 1b and 2). At this time, the disease symptoms were also strongly reduced in the P1-, P19-, AC2- and 2b-expressing transgenic plants. The height of these plants was about the same as that of the recovered non-transformed plants, but the number and size of the leaves were reduced compared with control plants, suggesting that they were not completely recovered. Fig. 1(d) shows the AC2 transgenic plant at 40 days p.i. The recovered phenotypes of the P1, P19 and 2b transgenic plants looked very similar to this (data not shown). The viral RNA level in each of these lines was reduced throughout the infection course (from 10 to 40 days p.i.), but not as much as in the non-transformed or in the pBin61-transformed plants (Fig. 2), confirming that the recovery was not as efficient as in the control plants. Only low levels of virus-specific siRNAs were detected in these plants both at 10 and 38 days p.i., correlating with the low viral RNA levels (Fig. 3).
In contrast to the non-transformed inoculated plants, the P25- and HcPro-expressing *N. benthamiana* plants continued to show systemic symptoms until the end of the experiment (40 days p.i.), with strongly reduced growth compared with the growth of the non-transformed infected plants (Fig. 1c), and also to the non-infected transgenic plants (data not shown), indicating that they did not recover from the infection. The lack of recovery was also confirmed by detection of a high level of viral RNA in the upper leaves of these plants at 40 days p.i. (Fig. 2). In addition, the level of TRSV-specific siRNA was high in the systemically infected leaves of these plants at both 10 and 38 days p.i., correlating with the high level of viral RNA (Fig. 3).

**TRSV infection in *N. tabacum* plants expressing various silencing suppressors**

The TRSV infection outcome in *N. tabacum* species was strongly dependent on the temperature. Under cooler growth conditions, at 18 °C, all tested non-transformed *N. tabacum* plants were susceptible to the virus. The infection produced severe systemic symptoms (Fig. 4a) and high levels of the viral RNA were detected in the systemic leaves at 35 days p.i. (Fig. 4b). The non-transformed plants continued to be susceptible to the virus up to the standard greenhouse temperature (25 °C). At this temperature, the non-transformed plants still became systemically infected, accumulating high levels of viral RNA at 35 days p.i. (Fig. 4b), but showed no or only mild symptoms in the systemically infected leaves (data not shown). However,

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**Fig. 1.** TRSV symptoms in *N. benthamiana* non-transformed (wt) and transgenic plants. (a) Typical symptoms in non-transformed plants at 10 days p.i. (b) Recovery of a non-transformed plant from initial infection at 40 days p.i. (c) Symptomatic and stunted HcPro- and P25-expressing transgenic plants compared with recovered non-transformed (wt) plants at 40 days p.i. (d) AC2-expressing transgenic plant showing small-sized leaves at 40 days p.i.

**Fig. 2.** Northern blot analysis of TRSV-inoculated *N. benthamiana* plants. (a) Viral RNA from samples of the uppermost leaves at 10 and 40 days p.i., probed with a TRSV-specific probe. (b) Ethidium bromide-stained rRNA is shown as a loading control. wt, Non-transformed control plant.

**Fig. 3.** TRSV-specific siRNA analysis from inoculated *N. benthamiana* plants. (a, c) Virus-specific siRNAs from samples of the uppermost leaves at 10 (a) and 38 (c) days p.i., probed with a TRSV-specific probe. (b, d) Ethidium bromide-stained rRNA is shown as a loading control. Marker, 25 nt 32P-labelled DNA oligonucleotide; Mock, mock infection; wt, non-transformed control plant.
when the plants were incubated at 33 °C (Fig. 4b), they did not develop any disease symptoms and no viral RNA was detected in the upper leaves at either 10 or 35 days p.i. (Fig. 4b).

The TRSV-inoculated transgenic *N. tabacum* plants, grown at 25 °C, accumulated viral RNA in the inoculated leaves as well as in the systemic leaves, basically to the same levels as the non-transformed plants (Fig. 5a and c). Mild ringspot and line pattern symptoms were occasionally observed in the control plants and also in the transgenic plants incubated at this temperature (data not shown). Thus, at this temperature, no significant difference was observed between non-transformed and transgenic plants or between different suppressor transgenic lines inoculated with TRSV.

When plants were grown at the temperature regime of 26–29 °C, viral RNA was detected in the inoculated leaves of the non-transformed plants and all of the suppressor-expressing transgenic lines (Fig. 6a). Nevertheless, no virus accumulation was detected in the systemic uppermost leaves of the control plants (non-transformed and pBin61 transgenics), either at 10 days p.i. (data not shown) or at 35 days p.i. (Fig. 6c), indicating that the systemic spread of the virus was restricted at this temperature. However, the HcPro- and AC2-expressing transgenic plants accumulated notably higher levels of viral RNA in the systemic leaves at 35 days p.i. (Fig. 6c). In three different experiments, with a total of eight tested plants each, seven of the HcPro-expressing plants and eight of the AC2-expressing plants were highly TRSV-positive at 35 days p.i. In addition, high levels of viral RNAs were also detected in the systemic leaves of some of the plants expressing P1 (2/8), P19 (3/8) and 2b (3/8) at 35 days p.i. In the other plants of these transgenic lines, only low or no viral accumulation was detected in the upper leaves at 35 days p.i. (Fig. 6c, and data not shown). In contrast to the *N. benthamiana* plants, no systemic accumulation of the virus was detected in any of the P25 transgenic *N. tabacum* plants. At this temperature regime, no symptoms were observed in any plant (data not shown).

**DISCUSSION**

The recovery phenomenon demonstrated here was described for the first time about 80 years ago (Wingard, 1928) and its molecular mechanism has been described as a manifestation of RNA silencing (Ratcliff *et al.* 1997). The susceptibility of the nepoviruses to this silencing-mediated defence suggests that these viruses do not produce effective silencing suppressors and, accordingly, their infections should be effectively enhanced by the silencing suppressors of other viruses.
Here, we tested the effects of six different viral silencing suppressors, expressed in two different transgenic hosts, *N. benthamiana* and *N. tabacum*, on infection of TRSV, potato calico strain. This aggressive TRSV strain was isolated from potato in South America and was known previously by the name potato black ringspot nepovirus (Fribourg et al., 1977; Harris et al., 2002). TRSV is known to infect a wide range of dicotyledonous plant species, including at least six different *Nicotiana* species (Brunt et al., 1996), and typically causes severe infections with various ringspot symptoms and with later recovery of the plants from the disease symptoms (Murant et al., 1996). In our hands, this strain caused a strong initial infection in *N. benthamiana* with distinct line pattern symptoms and ringspots, and later recovery of the plants from the infection. However, its infection pattern in *N. tabacum* depended strongly on the growth temperature of the inoculated plants. At cooler temperatures (18 °C), the plants were highly susceptible to systemic infection, accumulating high viral RNA levels and developing clear ringspots and line-pattern symptoms. At 25 °C, the plants developed systemic infections with mild or no symptoms. However, at 26 °C or higher temperatures the infections were limited to the inoculated leaves.

The infection pattern of TRSV, potato calico strain, varied significantly in the different silencing suppressor-expressing transgenic *N. benthamiana* lines. Initially, infection in all of the lines appeared very similar to infection in the wild-type plants. In the course of the infection, the non-transformed and pBin61-transformed control plants recovered from infection, with almost complete elimination of viral RNAs from the upper leaves. The viral RNA levels were also reduced in the P1-, P19-, AC2- and 2b-expressing transgenic lines, although not as much as in the non-transformed and pBin61-transformed control plants. The lack of virus-specific siRNAs in the recovered tissues apparently correlated with the small amounts of genomic viral RNAs in these tissues.

In contrast to the other transgenic lines, the HcPro- and P25-expressing plants exhibited enhanced initial virus accumulation, and both suppressors completely blocked the recovery of plants from infection. At 40 days p.i., the virus RNA was as abundant in the upper leaves of these plants as it was at 10 days p.i. The silencing appeared to be still active in the late infection stages, as indicated by the continuous accumulation of TRSV-specific siRNA. This accumulation of siRNAs in the systemically infected leaves correlated with the continuous presence of the viral RNAs (the substrate for siRNA synthesis). Similar correlations between virus-specific siRNAs levels and viral RNA levels have been reported previously (Szittya et al., 2002; Qu & Morris, 2005).

The different TRSV infection patterns observed in the HcPro- and P25-expressing plants appeared to be related to the different modes of action of these suppressors and, in particular, to their effects on the long-distance spread of the silencing signal. HcPro does not affect the long-distance spread of the systemic silencing signal (Mallory et al., 2001), but when constitutively expressed in the upper leaves and the shoot meristem, it apparently prevents the establishment and RDR6-mediated amplification of the systemic silencing (Schwach et al., 2005). Thus, the spreading virus was able to continuously establish new infection foci in the developing leaves, although local silencing was continuously activated in these tissues by the replicating virus. Via these processes, both viral RNA and siRNA accumulated in the systemically infected leaves, but the siRNAs failed to induce systemic silencing and recovery. The P25 suppressor, on the other hand, specifically prevents the long-distance silencing signal (Hamilton et al., 2002; Voinnet, 2005a), and may thus inhibit the induction of the systemic silencing status. Thus, the virus can freely spread and form new infection foci in the newly emerging leaves. Again, virus replication and active silencing were both activated in these tissues and balanced between each other to maintain the high levels of both viral RNA and virus-specific siRNAs.

When grown at a temperature of 25 °C, the wild-type *N. tabacum* plants developed systemic TRSV infection, and at this temperature the different viral silencing suppressors did not significantly affect virus spread or accumulation in this host. At 26 °C or higher temperatures, the plants could naturally suppress the systemic spread of the virus and prevent systemic infection. Interestingly, in the presence of HcPro or AC2 silencing suppressor transgenes, the plants were always fully susceptible to systemic infection at this temperature. The other suppressor transgenes (except for P25) also allowed accumulation of viral RNA in the systemically infected leaves, although this happened only occasionally. These data indicate that, under restrictive conditions, systemic virus spread was efficiently prevented in the non-transformed plants by a silencing-mediated defence. Thus, higher temperatures enhanced the silencing-mediated defence, which is in accordance with the results of Szittya et al. (2003) and Shams-Bakhsh et al. (2007).

It was of interest to compare the effects of the different silencing suppressors on TRSV infection in the two *Nicotiana* species. The HcPro transgene prevented plant recovery and allowed strong systemic infection in both species, although the siRNA analysis from *N. benthamiana* indicated that the local silencing continued throughout the infection. The P25 transgene also prevented recovery of *N. benthamiana* indicated that the local silencing continued throughout the infection. The P25 transgene also prevented recovery of *N. benthamiana* from systemic TRSV infection, but it did not allow the systemic infection of TRSV in the transgenic *N. tabacum* host at elevated temperatures. This failure in this *Nicotiana* species was probably due to the low expression level of the transgene in this line (Siddiqui et al., 2008). On the other hand, it is conceivable that the P25 protein function itself is temperature-sensitive, as indicated by the results of Close (1964) and Xie et al. (2001). The effects of the AC2 transgene also varied in the two *Nicotiana* species, i.e. it did not affect recovery from TRSV infection in *N. benthamiana*, whereas it allowed a
high level of accumulation of the virus in the systemic leaves of N. tabacum at restrictive temperatures. AC2 is known to be a transcription activator, whose suppressor activity functions through modification of the host transcriptome (Trinks et al., 2005); therefore, it is not surprising that its effect depends on the host species.

It was also of interest that the P1, P19 and 2b suppressors affected TRSV infection only partially in both Nicotiana species. P1 of RYMV has been shown to prevent the systemic spread of the silencing signal in a sense-transgene system in N. benthamiana (Hamilton et al., 2002; Himber et al., 2003). In those studies, P1 was expressed transiently and not transgenically, and it is possible that its weak effect observed in the TRSV infections could be due to the different experimental settings used in these two experiments. P19 binds and sequesters siRNA duplexes (Vargason et al., 2003) and is therefore partially able to block systemic silencing. However, if amplification of silencing produces too many secondary siRNAs, P19 may not be able to sequester all of them and this explains the partial effect on TRSV infection in both Nicotiana species. The 2b silencing suppressor interferes with the long-distance spread of the silencing signal (Guo & Ding, 2002) and interacts directly with AGO1 (Zhang et al., 2006). It should be noted, however, that the suppressor efficiency of 2b has been reported to depend on the CMV strain it is derived from (Goto et al., 2007; Lewsey et al., 2007). The 2b gene used in this work was derived from the mild Kin strain of CMV, which might explain the weak effect observed.

Interestingly, seed transmission appears to be an important survival feature for the nepoviruses. This is mediated via their ability to invade meristematic tissues and the germ cells of their hosts, which in most viral infections appear to be prevented by silencing reactions, induced by RDR6-mediated amplification of the systemic silencing signals in the meristematic cells (Foster et al., 2002; Schwach et al., 2005). It is possible that the recovery-related low accumulation of the nepoviruses in the vegetative tissues actually facilitates entry to the meristem. In this work, we have shown that accumulation of the siRNAs, and thus also of the transmittable silencing signal, is very low in the recovered tissues (in this study, it was below the detection level). RDR6 activity is known to be induced only by high target RNA levels (Schwach et al., 2005; Voinnet, 2005b), and thus a low level of inducing signal may fail to activate RDR6-mediated systemic silencing in the meristematic tissue. Thus, meristem entry may be feasible only to those viruses that can prevent either the high accumulation or spread of the silencing signal.

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