INTRODUCTION

RNA silencing, also known as co-suppression and post-transcriptional gene silencing, is a sequence-specific RNA degradation mechanism first discovered in transgenic plants (Napoli et al., 1990; Smith et al., 1990; van der Krol et al., 1990). Similar phenomena have been described in diverse eukaryotic organisms including fungi, where it is known as quelling, and animals, in which it is referred to as RNA interference, and have been the subject of intensive research over the last few years (for reviews, see Cogoni, 2001; Hannon, 2002; Zamore, 2002).

Although RNA silencing in plants has been studied most extensively using transgenes, viruses can be both initiators and targets of RNA silencing (Ratcliff et al., 1997; Voinnet et al., 1999). RNA silencing mechanisms derived from virus infection (virus-induced gene silencing, VIGS) and from transgenes are closely related and appear to share many components, but have been shown to use different pathways to generate the double-stranded RNA that triggers the process (Dalmay et al., 2000; Bécina et al., 2002; Morel et al., 2002). Many data obtained in recent years support RNA silencing as an inducible host RNA surveillance mechanism used for defence against virus infection and transposon-induced abnormalities in gene expression, thus allowing the plant to recognize and destroy foreign or aberrant RNAs (for reviews, see Vance & Vaucheret, 2001; Voinnet, 2001; Waterhouse et al., 2001; Vazquez Rovere et al., 2002).

The study of this intriguing defence mechanism of plants against virus infection has led to the discovery of virus-encoded suppressors of RNA silencing (Anandalakshmi et al., 1998; Bécina et al., 1998; Brigneti et al., 1998; Kasschau & Carrington, 1998). The ability of plant viruses to suppress RNA silencing is probably part of a counter-defensive strategy to overcome the silencing response (for reviews, see Carrington et al., 2001; Baulcombe, 2002). Silencing suppressors which have distinct modes of action have been described in diverse DNA and RNA viruses of plants (Voinnet et al., 1999). The first viral suppressor of silencing characterized was the HCPro protein encoded in the potyviral genome, which has been shown to interfere with both transgene-induced RNA silencing (Anandalakshmi et al., 1998; Kasschau & Carrington, 1998) and VIGS (Anandalakshmi et al., 1998; Brigneti et al., 1998). HCPro acts by blocking the maintenance of RNA silencing in tissues where silencing has already been established. In contrast, the 2b protein of cucumoviruses is unable to reverse already established RNA silencing, but prevents its initiation at the growing points of the plant by inhibiting the long-range activity of the silencing signal produced during the silencing reaction (Bécina et al., 1998; Brigneti et al., 1998; Guo &
Ding, 2002). Other suppressors, such as the p25 protein of the potexviruses, also affect RNA silencing by preventing spreading of the silencing signal (Voinnet et al., 2000).

RNA silencing of transgenes homologous to viral sequences has been shown to give rise to highly specific resistance against the virus from which the transgene derives (Lindbo & Dougherty, 1992; Mueller et al., 1995). Sometimes, the transgene is constitutively silenced and the transgenic plant is immune to virus infection. However, in other cases, transgene silencing and the consequent establishment of resistance must be induced by an initial virus infection, resulting in a ‘recovery’ phenotype. The recovered tissues are free of viral RNA and proteins and are resistant to superinfection by the same virus, but susceptible to infection by unrelated viruses (Lindbo et al., 1993). Recovery from virus disease is not restricted to transgenic plants and can also be activated naturally in some virus infections. In these cases, the infected plants exhibit a response very similar to the virus-induced recovery of transgenic plants in that the upper leaves are symptom free, contain reduced levels of virus and exhibit a strong and specific virus resistance (Covey et al., 1997; Ratcliffe et al., 1997).

We have constructed transgenic Nicotiana benthamiana plants transformed with a genome fragment of Plum pox virus (PPV), a member of the Potyvirus genus (López-Moya et al., 2000), which show a phenotype of delayed resistance mediated by RNA silencing (Guo & García, 1997; Guo et al., 1999). In the work described in this paper, the paradox that an infection producing an efficient silencing suppressor (HCPro) can induce RNA silencing is approached by assessing the suppression of the induced silencing and the consequent delayed virus resistance following infection with two heterologous viruses producing related and unrelated silencing suppressors.

METHODS

**Virus and transgenic plants.** The plum pox potyvirus Rankovic isolate (PPV-R) and the transgenic N. benthamiana line NbV3, transformed with a fragment of the PPV-R genome containing the 5′NTR and the NbV coding sequence with a Gly→Val mutation, have been described previously (Guo & García, 1997). Tobacco vein mottling virus (TVMV) and the Fny strain of Cucumber mosaic virus (CMV) were provided by E. Rodríguez-Cerezo (Centro Nacional de Biotecnología, Madrid, Spain) and F. García-Arenal (Universidad Politécnica, Madrid, Spain), respectively.

Transgenic plants were grown from seeds germinated in the presence of kanamycin at a concentration of 0·1 mg ml⁻¹.

**Plant regeneration.** Plants were regenerated from sterilized leaf disks by *in vitro* culture on solid Murashige and Skoog (MS) medium containing 6-benzylaminopurine (1 mg l⁻¹), α-naphthaleneacetic acid (0·1 mg l⁻¹) and kanamycin (0·1 mg ml⁻¹). Regenerated shoots were rooted on MS medium without hormones. Rooted plantlets were transferred to soil and grown in a climate-controlled room at 60% relative humidity in a 14 h light (22°C) and 10 h dark (20°C) cycle.

**Inoculation and sampling.** Young (five-leaf stage) plants were inoculated by rubbing inocula on to three leaves dusted with Carborundum. Crude sap from Nicotiana clevelandii plants infected with PPV or N. benthamiana plants infected with CMV or TVMV (1 g in 2 ml 5 mM sodium phosphate, pH 7·2) was used as the source of inoculum for these viruses. In the co-inoculation experiments, the two extracts were mixed just before being applied to the Carborundum-dusted leaves. For sequential inoculation, the first virus was inoculated as described above and, 2–4 weeks later, one leaf with symptoms immediately above those first inoculated was sampled to analyse transgene expression; the second virus was then inoculated on to the following leaf (also showing symptoms). Plants were maintained in a climate-controlled room at 60% relative humidity in a 14 h light (22°C) and 10 h dark (20°C) cycle. PPV accumulation was assessed at different days post-inoculation by double-antibody sandwich indirect ELISA with the REALISA kit (Durviz), and TVMV and CMV accumulation was assessed by ELISA with antibodies obtained from the ATCC. Samples consisted of single discs of 7 mm diameter collected from leaves situated above the inoculated ones.

**Analysis of RNA.** Total RNA was isolated from leaf tissue by the LiCl precipitation method described by Verwoerd et al. (1989). For Northern blot analysis, total RNA (10 μg) was separated on a 1·2% agarose gel containing 6% formaldehyde and transferred and UV cross-linked (1200 mJ, Stratalinker; Stratagene) to a Zeta-probe membrane (Bio-Rad) (Sambrook et al., 1989). The blot was hybridized with a 32P-labelled riboprobe specific for the PPV Nib coding sequence, synthesized by *in vitro* transcription with MAXIscript kit (Ambion). Methylene blue staining of the membrane after blot transfer was used to show the ribosomal RNA (rRNA) loading (Sambrook et al., 1989). Both hybridization signals and rRNA content were quantified with a phosphorimager (model PSI-486; Molecular Dynamics) and these values were used to calculate the relative amounts of transgene mRNA in each sample.

RESULTS

Previous studies have shown that transgenic N. benthamiana NbV3 plants, which are transformed with a PPV Nib coding sequence carrying a Gly→Val mutation at the GDD motif typical of viral RNA replicases, recover from an initial PPV infection and become highly resistant to PPV by the VIGS mechanism (Guo & García, 1997). It is remarkable that recovery starts in infected plants producing HCPro, a protein described as an efficient silencing suppressor, which is maintained in the upper part of the plant even when the infection is still active in the lower leaves (Guo & García, 1997).

In order to assess the susceptibility of the VIGS established in the recovered NbV3 plants to the suppressor activity of different viral proteins, we regenerated plantlets from asymptomatic leaf explants in *in vitro*. Both transgene silencing and resistance to PPV were maintained in these plants in the absence of the inducer PPV infection (Guo et al., 1999 and Fig. 1). They will subsequently be referred to as ‘recovered’ plants.

**CMV infection suppresses transgene silencing and PPV resistance in recovered NbV3 plants**

In order to assess whether the transgene silencing of NbV3, which had been induced in the presence of HCPro, could be reverted by silencing suppressor(s) of CMV,
recovered NiBv3 plants were inoculated with this virus. Northern blot analysis showed that CMV infection caused a clear increase in the accumulation levels of the PPV transgene in the silenced plants (Fig. 2A, B).

To verify whether silencing suppression by CMV was associated with resistance breaking, CMV and PPV were inoculated either simultaneously or sequentially on to NiBv3 recovered plants. Virus accumulation on leaves above the inoculated ones was assessed by ELISA (Table 1). All recovered plants co-inoculated with both viruses were infected with CMV, but none of them was infected with PPV, in contrast to the high rate of PPV infection in the co-inoculated untransformed plants used as controls (Table 1). However, when the silenced plants were sequentially inoculated, first with CMV and then with PPV, the number of NiBv3 recovered plants that became infected with PPV was similar to that obtained when untransformed N. benthamiana plants were inoculated in the same way (Table 1). PPV was detected in the CMV/PPV co-infected NiBv3 plants for at least 1 month after inoculation and virus accumulation was similar to that obtained in the untransformed controls. No new cycles of recovery were observed in any of the nine co-infected recovered plants for at least 1 month.

Although the percentage of CMV-infected plants that were susceptible to PPV infection was similar for recovered and untransformed plants, since not all CMV-infected plants became infected with PPV, the possibility exists that a certain level of silencing suppression might be necessary to overcome the resistance to PPV infection in the recovered plants. Northern blot analysis was carried out to determine the steady-state levels of transgene mRNA accumulation at the time of PPV challenge in the CMV-infected plants that became infected with PPV and in those that remained uninfected (Fig. 3 and data not shown). No transgene mRNA was detected before CMV inoculation in any of the 15 recovered plants analysed. After CMV infection, transgene silencing was suppressed to various degrees in all plants. Differences in the levels of transgene mRNA accumulation were detected even among different leaves from the same plant (see, for example, plant #21 in Fig. 3). However, after phosphorimager quantification of the hybridization signals, no correlation was observed between higher transgene silencing suppression and susceptibility to PPV. It was also noticeable that in the 23 leaves from the 15 CMV-infected silenced plants analysed the transgene mRNA accumulation never reverted to the high steady-state levels reached in the non-infected transgenic NiBv3 plants (Fig. 3 and data not shown). Thus, it did not seem to be necessary to suppress completely the transgene silencing established in the recovered plants in order to break down virus resistance.

![Fig. 1](http://vir.sgmjournals.org 2879)

Fig. 1. (A) Schematic representation of the experimental procedure followed to regenerate silenced plants from recovered tissue from PPV-infected NiBv3 plants. (B) Transgene mRNA accumulation in non-infected and recovered tissue of NiBv3 plants and in *in vitro*-regenerated recovered plants. Total RNA (~ 10 µg) was blotted and subjected to hybridization with a 32P-labellled probe specific for the NiB sequence (upper panel). Methylene blue staining of the membrane after blot transfer was used to check loading of RNA samples (lower panel).
TVMV infection suppresses transgene silencing but does not restore PPV susceptibility in recovered NiBV3 plants

Since NiBV3 plants recover when HCPro is being actively produced, it is tempting to speculate that the transgene silencing of the recovered NiBV3 plants might be resistant to the suppressor activity of the potyviral HCPro. To test this hypothesis, recovered NiBV3 plants were inoculated with another potyvirus, TVMV. Northern blot analysis showed that TVMV infection strongly suppressed the transgene silencing of the NiBV3 recovered plants even more efficiently than CMV infection (Fig. 2A, B). However, TVMV infection did not appear to be able to break down the PPV-resistance of the NiBV3 recovered plants in joint inoculations. Although TVMV was detected by ELISA in the systemic leaves of all TVMV/PPV co-inoculated plants and PPV was able to infect a large proportion of non-transgenic N. benthamiana plants co-inoculated with both potyviruses, PPV accumulation was never detected in recovered NiBV3 plants infected with TVMV, regardless of whether PPV was applied together with TVMV or whether its inoculation was delayed to leaves with noticeable symptoms of TVMV infection (Table 1).

**DISCUSSION**

It is well established that post-transcriptional silencing of viral transgenes leads to resistance specific for the virus from which the transgene derives (Waterhouse *et al.*, 1999). Both transgene silencing and resistance to the homologous virus have been shown to be overcome by infection with another unrelated virus (Mitter *et al.*, 2001; Savenkov & Valkonen, 2001). In this work we have approached the question of the susceptibility of RNA silencing and the subsequent virus resistance, induced by a virus infection that produces a
Both CMV, which produces the potyvirus-unrelated silencing suppressor 2b, and the potyvirus TVMV suppressed the PPV-induced silencing of the recovered NiðV3 plants (Fig. 2). This indicated that, although the silencing of the PPV transgene is induced by an infection that produces HCPro, it is still susceptible to the suppressor activity of a presumably similarly acting potyviral protein. Two main conclusions can be drawn from this result: (i) since virus replication and thus HCPro accumulation is maintained in the primary infected leaves in the recovering NiðV3 plants, the silencing suppression activity of HCPro must not be able to act at a distance; and (ii) HCPro cannot interfere with the propagation of silencing signals, which induce transgene silencing and virus resistance in the upper leaves of the NiðV3 plants before PPV can arrive, replicate and then produce HCPro.

There are conflicting data on the capacity of HCPro to interfere with the systemic silencing signal. Using grafting experiments and transgenic expression of both HCPro and the silencing inducer, Mallory et al. (2001) showed that HCPro was unable to block spreading of the silencing signal. In contrast, transient expression by agroinfiltration of HCPro was shown to prevent the systemic spread of silencing of a GFP transgene induced by a homologous construct also expressed by agroinfiltration (Hamilton et al., 2002; Pfeffer et al., 2002). The apparent inconsistency of these experiments probably rises from the different transgenes and expression systems used (Voïnnet, 2001). It is likely that HCPro has some ability to disturb the spreading of the systemic silencing signal, either by direct interference with it or by causing a decline in its production. However, the effectiveness of the blockade of the silencing spread would depend on the quantitative balance between HCPro and the silencing signal produced in each case. In contrast with the previous approaches, in our experimental system recovery of the NiðV3 plants took place in the context of a potyvirus infection, indicating that in such a natural situation, HCPro is unable to block the systemic propagation of the silencing signal successfully in advance of the virus infection.

Interestingly, suppression of the Nið transgene silencing caused by CMV infection but not by TVMV infection restored susceptibility to PPV in the recovered NiðV3 plants (Table 1). The inability of TVMV infection to reverse the resistance to PPV is not likely to be due to inefficient silencing suppression since: (i) CMV infection facilitated PPV infection of the recovered NiðV3 plants, although it never reverted the transgene mRNA accumulation levels to those of the transgenic plant prior to the initial PPV infection (Fig. 2); (ii) no apparent correlation between higher transgene mRNA accumulation and PPV susceptibility was observed in recovered NiðV3 plants inoculated first with CMV and then with PPV (Fig. 3); and (iii) TVMV appears to suppress the silencing of the NiðV3 plants more efficiently than CMV. At least two possible explanations could account for our results. The first involves an interpotyvirus cross-protection mechanism. Although no cross protection of TVMV against PPV was observed in

silencing suppressor, to the activity of related and non-related viral silencing suppressors.

We used the N. benthamiana line NiðV3 (Guo & García, 1997). This line is transformed with a PPV transgene that becomes silenced after an initial PPV infection. We studied the effect of infection with a second virus in plants regenerated from the recovered tissue (‘recovered’ plants), rather than in the primarily infected and recovered plants. These ‘recovered’ plants retained the silencing of the transgene and the PPV-resistance phenotype but were completely free of the infecting virus (Fig. 1B). Thus, they allowed us to study the result of the second virus infection on juvenile tissue without possible interference of the silencing suppressor from the first infection.

Both CMV, which produces the potyvirus-unrelated silencing suppressor 2b, and the potyvirus TVMV suppressed the

Fig. 3. Analysis of the transgene silencing suppression of CMV-infected recovered NiðV3 plants differing in their susceptibility to PPV infection. Sixteen recovered plants were sequentially inoculated with CMV and PPV (numbering of these plants is independent from that of the recovered plants described in Fig. 2). (A) Northern blot analysis of total RNA samples (~10 μg) extracted from leaves of some of the plants immediately before CMV inoculation (–) or from one or two uppermost systemic leaves 16 days after CMV inoculation and before PPV challenge (C1 and C2). Plants 20 and 21 did not become infected by PPV (PPV–) while plants 13, 18 and 31 were infected (PPV +). Samples from a non-PPV-infected NiðV3 plant and from a non-transgenic (NT) N. benthamiana plant are shown as controls. The position of the transgene is indicated. Methylene blue staining of the membrane after blot transfer was used to check loading of RNA samples (lower panels). (B) Quantification of the hybridization signals corrected for the amount of loaded RNA was accomplished using a phosphorimaget.
non-transgenic PPV systemic hosts such as N. benthamiana (Table 1) or N. clevelandii (data not shown), the infection of another potyvirus, Tobacco etch virus, has been shown to interfere with PPV replication in Nicotiana tabacum, a local host for PPV (Sáenz et al., 2002). Thus, the possibility exists that cross protection is only apparent when PPV replication is weakened, either by an unsuitable host, such as tobacco, or by some RNA silencing activity still remaining in the TVMV-infected recovered NibV3 plants. In this scenario, the susceptibility to Potato virus A (PVA) after Potato virus Y infection of N. benthamiana plants transformed with a constitutively silenced PVA coat protein transgene (Savenkov & Valkonen, 2001) could be explained by a less efficient cross protection in this virus/host/transgene system.

Alternatively, the inconsistency between transgene silencing suppression and resistance breakdown in the virus-infected recovered NibV3 plants could be revealing the existence of more than one silencing mechanism induced by the former PPV infection, which would have different susceptibility to the suppressor activities of CMV 2b and TVMV HCPo. At least two branches have been recognized in the induction pathway of RNA silencing in plants (Voinnet et al., 2000; Béclin et al., 2002; Mlotshwa et al., 2002). In addition, the possible existence of two separate mechanisms for silencing-related target mRNA destruction has recently been suggested (Tang et al., 2003). Our results could be explained by assuming that both the constitutively PVA-resistant plants described by Savenkov & Valkonen (2001) and the recovered NibV3 plants have an RNA silencing mechanism that is suppressible by both HCPo and 2b, while a second silencing mechanism, specific for target degradation of viral RNA, is only induced by the virus-induced silencing of the recovered plants and would be efficiently suppressed by 2b but not by HCPo. We also cannot rule out the possibility that PPV infection induces non-silencing-related defence mechanisms in the recovered NibV3 plants, which would be not active in the constitutively immune transgenic plants and which could be counteracted by CMV but not by TVMV infection. In this regard, it is important to note that CMV 2b has been shown to be involved not only in silencing-related defence mechanisms but also in virus resistance mediated by a hypersensitive response (Li et al., 1999; Ji & Ding, 2001).

The mechanisms leading to RNA degradation through RNA silencing remain unclear at both the cellular and the plant level. Numerous models have been proposed but none fully account for the broad range of phenomena described. Further characterization of the recovery process will contribute to a better understanding of virus resistance and RNA silencing in plants and will help to design more effective and durable strategies to confer virus resistance to plants.

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