Journal of General Virology (2003), **84**, 2877–2883 DOI 10.1099/vir.0.19263-0

Suppressor activity of potyviral and cucumoviral infections in potyvirus-induced transgene silencing

Carmen Simón-Mateo, Juan José López-Moya,† Hui Shan Guo,‡ Elena González and Juan Antonio García

Centro Nacional de Biotecnología (C.S.I.C.), Campus de la Universidad Autónoma de Madrid, 28049 Madrid, Spain

Correspondence
Juan Antonio García
jagarcia@cnb.uam.es

Received 1 April 2003
Accepted 24 May 2003

The process known as 'recovery' by which virus-infected plants become resistant to the infection is an interesting phenomenon where both RNA silencing and virus resistance fully converge. In a previous study, we showed that transgenic *Nicotiana benthamiana* NIbV3 plants, transformed with a mutated NIb coding sequence from *Plum pox virus* (PPV), showed a delayed, very specific, resistance phenotype, which was induced by the initial infection. This recovery was the consequence of the activation of an RNA silencing mechanism in the PPV-infected plant, which took place even though PPV encodes a silencing suppressor (HCPro). Making use of plants regenerated from the recovered tissue, which maintained the transgene silencing/virus resistance phenotype, we have demonstrated that both *Cucumber mosaic virus* (CMV) and *Tobacco vein mottling virus* (TVMV), expressing the silencing suppressor 2b and HCPro, respectively, were able to reactivate transgene expression. Surprisingly, only the silencing suppression caused by CMV, but not that originating from TVMV, was able to revert the recovered NIbV3 plants to a PPV-susceptible phenotype.

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; Béclin *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; Béclin *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 (Béclin *et al.*, 1998; Brigneti *et al.*, 1998; Guo &

†Present address: Centro de Investigaciones Biológicas (C.S.I.C.), Velázquez 144, 28006 Madrid, Spain.

‡Present address: Institute of Molecular Agrobiology, 1 Research Link, The National University of Singapore, Singapore 117604.

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; Ratcliff *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 NIbV3, transformed with a fragment of the PPV-R genome containing the 5′NTR and the NIb 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-naphthalene acetic 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 ^{32}P-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* NIbV3 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 NIbV3 plants to the suppressor activity of different viral proteins, we regenerated plantlets from asymptomatic leaf explants *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 NIbV3 plants

In order to assess whether the transgene silencing of NIbV3, which had been induced in the presence of HCPro, could be reverted by silencing suppressor(s) of CMV,

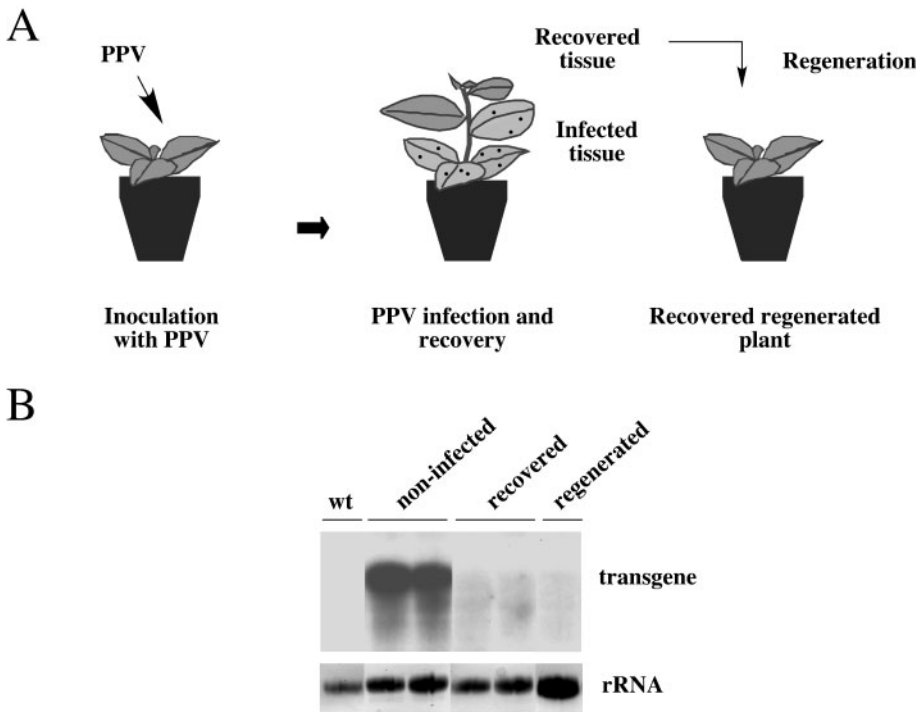

Fig. 1. (A) Schematic representation of the experimental procedure followed to regenerate silenced plants from recovered tissue from PPV-infected NIbV3 plants. (B) Transgene mRNA accumulation in non-infected and recovered tissue of NIbV3 plants and in *in vitro*-regenerated recovered plants. Total RNA (~10 μg) was blotted and subjected to hybridization with a ^{32}P-labelled 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).

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.

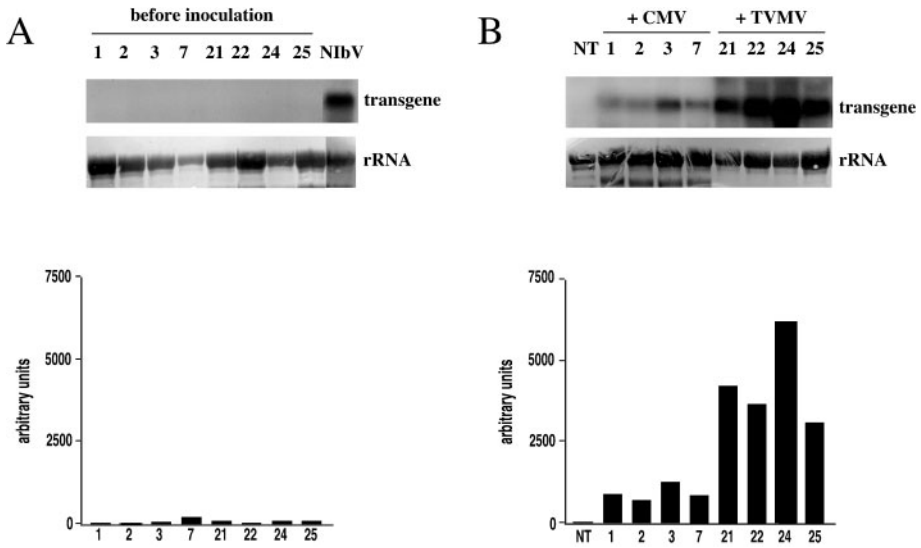

Fig. 2. Suppression of transgene silencing following CMV and TVMV infection of NIbV3 recovered plants. Ten recovered plants were infected with each virus in this experiment. Upper panels show Northern blot analysis from total RNA samples (~10 μg) extracted from leaves of some recovered plants (#1, 2, 3, 7, 21, 22, 24 and 25) immediately before inoculation (A) and 15 days after CMV (#1, 2, 3 and 7) or TVMV (21, 22, 24 and 25) inoculation (B). RNA from a non PPV-infected NIbV3 plant and from a non-transgenic (NT) *N. benthamiana* plant was also analysed. The position of the transgene mRNA is indicated. Methylene blue staining of the membrane after blot transfer was used to check loading of RNA samples (lower panel). Quantification of the hybridization signals corrected for the amount of loaded RNA was accomplished using a phosphorimager (graphs).

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).

Table 1. Detection of PPV, CMV and TVMV by ELISA in the upper inoculated leaves of recovered NIbV plants and non-transgenic *N. bethamiana*

Results are shown as no. of infected plants/no. of inoculated plants.

	N. benthamiana		Recovered NIbV	
(a) CMV + PPV	CMV	PPV	CMV	PPV
Co-inoculated	5/5	5/5	8/8	0/8
Sequentially inoculated	8/8	6/8	16/16	9/16

	N. benthamiana		Recovered NIbV	
(b) TVMV + PPV	TVMV	PPV	TVMV	PPV
Co-inoculated	4/4	4/4	9/9	0/9
Sequentially inoculated	9/9	5/9	10/10	0/10

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

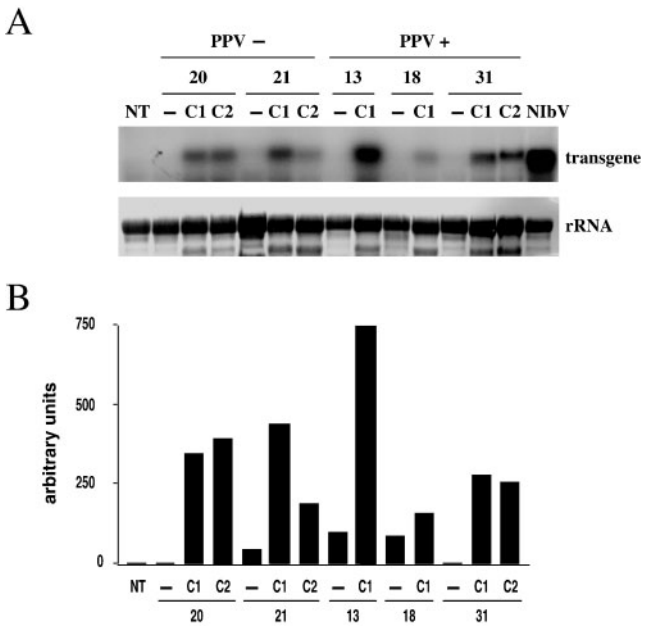

Fig. 3. Analysis of the transgene silencing suppression of CMV-infected recovered NIbV3 plants differing in their susceptibility to PPV infection. Sixteen recovered plants were sequentially inoculated with CMV and PPV (numbering of these plants is independent from that of the recovered plants described 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 NIbV3 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 phosphorimager.

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

We used the *N. benthamiana* line NIbV3 (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 PPV-induced silencing of the recovered NIbV3 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 NIbV3 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 NIbV3 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 (Voinnet, 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 NIbV3 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 NIb transgene silencing caused by CMV infection but not by TVMV infection restored susceptibility to PPV in the recovered NIbV3 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 NIbV3 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 NIbV3 plants inoculated first with CMV and then with PPV (Fig. 3); and (iii) TVMV appears to suppress the silencing of the NIbV3 plants more efficiently than CMV. At least two possible explanations could account for our results. The first involves an inter-potyvirus cross-protection mechanism. Although no cross protection of TVMV against PPV was observed in

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 HCPro. 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 HCPro 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 HCPro. 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.

ACKNOWLEDGEMENTS

We wish to thank Elvira Domínguez for technical assistance. C. S.-M. is a recipient of a post-doctoral fellowship from the Comunidad de Madrid. This work was supported by Grants BIO2001-1434 from CICYT and QLG2-2002-01673 and QLK2-2002-01050 from the European Union.

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