

RESISTANCE TO *PLUM POX VIRUS* (PPV) IN *NICOTIANA BENTHAMIANA* PLANTS TRANSFORMED WITH THE PPV HC-PRO SILENCING SUPPRESSOR GENE

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SUMMARY

Resistance to *Plum pox virus* (PPV) was tested in transgenic *Nicotiana benthamiana* plants containing a translatable construct derived from the full-length helper-component proteinase (HC-Pro) gene of PPV. Some of the lines tested showed a resistance characterized by a lack of systemic symptoms and viral RNA accumulation in systemic and inoculated leaves. Resistance was also effective against another closely related PPV strain, but not against other potyviruses such as *Tobacco etch virus* (TEV) and *Potato virus Y* (PVY). Resistance to PPV correlated with a lower accumulation of HC-Pro transgene mRNA and the accumulation of short-interfering RNAs (siRNAs) derived from the transgene. Resistance to PPV also correlated with the trans-inactivation of a transiently expressed GUS gene, which shares homology with the 3' portion of the HC-Pro transgene. These results indicate that the PPV resistance was caused by a post-transcriptional gene silencing (PTGS) process promoted by the HC-Pro transgene, which can induce silencing under certain conditions despite encoding a suppressor of PTGS.

Key words: Post-transcriptional gene silencing, transgenic resistance, silencing suppressor, PPV, HC-Pro.

INTRODUCTION

Transformation of plants with virus-derived sequences has been exploited for engineering disease resistance in many virus-host combinations (Beachy, 1997; Goldbach *et al.*, 2003). In most situations, resistance involves post-transcriptional gene silencing (PTGS), a specific RNA degradation mechanism related to 'quelling' in fungi and 'RNA interference' in animals (Waterhouse *et al.*, 2001; Hannon, 2002; Cerutti, 2003). PTGS triggered by a transgene causes specific degrada-

tion of the transgenic RNA and other RNAs containing enough sequence homology to the transgene, and produces accumulation of cognate short-interfering RNAs (siRNAs). Silencing of transgenes containing virus-derived sequences can cause resistance against viruses homologous to the transgene, a phenomenon termed homology-dependent resistance (HDR) (Baulcombe, 1996).

Most viruses can trigger PTGS as well as being targeted by it (Ruiz *et al.*, 1998; Marathe *et al.*, 2000). This can lead to a 'recovery' phenotype in transgenic plants, characterized by initial infection followed by a subsequent decrease in virus accumulation and symptoms in upper leaves as well as degradation of both viral RNA and transgene RNA (Lindbo *et al.*, 1993; Guo and García, 1997). Recovery can happen with some viruses even in the absence of homologous transgenes (Covey *et al.*, 1997; Ratcliff *et al.*, 1997). This observation suggests that PTGS may serve as a natural defence mechanism against viruses and other invading RNAs such as transposons (Voinnet, 2001; Waterhouse *et al.*, 2001). Further evidence supporting this view is that many viral pathogenicity determinants required for virus infection have been demonstrated to interfere with PTGS mechanisms (Voinnet *et al.*, 1999; Moissiard and Voinnet, 2004; Roth *et al.*, 2004). Among these are the HC-Pro of several potyviruses, which have been shown to interfere with PTGS promoted by transgenes and viruses in *N. benthamiana* and other host plants (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau and Carrington, 1998) and also to be involved in the PTGS-related phenomenon of viral synergism (Pruss *et al.*, 1997). The HC-Pro of PPV, an economically important potyvirus that typically infects woody trees in nature, can also function as a gene silencing suppressor in *N. benthamiana* (Tenllado *et al.*, 2003). Suppression of PTGS by HC-Pro is not always complete, it can be overcome by strong elicitors of PTGS, such as transgenes producing long segments of double-stranded RNA (dsRNA) and replicating RNA viruses (Johansen and Carrington, 2001). The 'recovery' phenotype in transgenic plants also involves silencing of the virus and the homologous transgene in the presence of HC-Pro in the case of potyviruses, or other suppressors of PTGS in the case of other viruses. In the present work we explore the role of

the PPV HC-Pro transgene in inducing PTGS and virus resistance while encoding a suppressor of PTGS.

MATERIALS AND METHODS

Virus strains. *Plum pox virus* (PPV) isolates 5.15 and 3.3 were used in this study and have been previously described (López-Moya *et al.*, 1994). *Potato virus Y* (PVY) strain 0-AT was described (Canto *et al.*, 1995). *Tobacco etch virus* (TEV) strain HAT was produced by inoculating RNA transcripts from the cDNA clone pTEV7DA (Dolja *et al.*, 1992) in *N. tabacum* cv. Xanthi, and using the infected plants as source of inoculum.

Plasmid constructs. A DNA fragment containing the HC-Pro gene of PPV 5.15 [nucleotides 1068 to 2444 of the PPV sequence (Laín *et al.*, 1989)] was amplified by Polymerase chain reaction (PCR) using a cDNA clone as template. The upstream primer was 5' AAATCTA-GAATGACTCTGACCCAGGC 3' (in italics nucleotides 1068 to 1082 of the PPV sequence), which introduces an *Xba*I site (underlined) and a translational start codon (bold). The downstream primer was 5' GAAGGATCCTTATCCAACCAGGTATGT 3' (in italics nucleotides complementary to 2430 to 2444 of the PPV sequence), which introduces a *Bam*HI site (underlined) and a stop codon (bold). The PCR product was digested with *Xba*I and *Bam*HI, and cloned into pT3T7 (Roche Diagnostic, Indianapolis, USA). Once the presence of the modified nucleotides and the lack of undesired changes were confirmed by sequencing, the HC-Pro fragment was cloned into the binary vector pBI121 (Jefferson *et al.*, 1987). This vector contains an *npt*II gene as a selective marker and a GUS gene under the control of a cauliflower mosaic virus (CaMV) 35S promoter. The HC-Pro coding sequence was inserted between the 35S promoter and the GUS coding sequence producing pBI-HCT. The GUS sequence retains its initiation codon, however it is unlikely to be translatable at high efficiency because of the presence of an in-frame stop codon upstream. Plasmid pBI/54-kDa was produced by removing the GUS sequence from pBI121 and inserting a DNA fragment comprising part of the 54-kDa-protein gene of pepper mild mottle virus [PM-MoV; nucleotides 3411 to 4388, (Alonso *et al.*, 1991)]. pBI121, pBI-HCT and pBI/54-kDa were transferred to *Agrobacterium tumefaciens* strain LBA4404 by heat-shock transformation.

Transformation of *N. benthamiana*. Transformation of *N. benthamiana* was performed using the leaf disk transformation method described by Horsch *et al.* (1985). Transformed shoots were regenerated in Murashige-Skoog medium (MS) supplemented with 6-benzylaminopurine (1 mg l⁻¹), α -naphthaleneacetic acid

(0.1 mg l⁻¹), kanamycin (100 mg l⁻¹) and cefotaxime (500 mg l⁻¹). Shoots were rooted in MS medium supplemented with indole-3-butyric acid (0.5 mg l⁻¹), kanamycin (25 mg l⁻¹) and cefotaxime (500 mg l⁻¹). Resulting plantlets were transferred to soil and grown in an environmental control chamber. The transgenic status of regenerated plants was checked by PCR from plant DNA extracted with CTAB (cetyltrimethylammonium bromide) as previously described (McGarvey and Kaper, 1991). Regenerated plants (R₀) were self-fertilized and seeds were collected. Seeds were germinated in MS medium containing kanamycin (100 mg l⁻¹) and plantlets were grown for three weeks. Kanamycin-resistant plantlets (R₁) were transferred to soil and grown for approximately two weeks more.

Virus inoculations. Plants were inoculated by applying a stock inoculum to two leaves dusted with carborundum. Stock inocula of each virus were prepared by grinding infected plant tissue in five volumes of 20 mM sodium phosphate buffer (pH 7.0) and storing them in single-use aliquots at -80°C. Fresh inoculum of PPV 5.15 was prepared at the moment of use by grinding infected tissue in two volumes of sodium phosphate buffer. The infectivity of PPV 5.15 inocula were estimated by counting the local lesions induced in manually inoculated *Chenopodium foetidum* leaves.

Detection of HC-Pro by Western blotting. For HC-Pro detection in transgenic plants, samples of 100 mg of leaf tissue were ground first in liquid nitrogen and then in 100 μ l of extraction buffer (200 mM Tris-Cl, pH 6.8, 5% SDS, 5% β -mercaptoethanol, 10% glycerol, 0.005% bromophenol blue). Homogenized samples were boiled for 5 minutes, clarified by centrifugation and 15 μ l were separated by electrophoresis in 10% polyacrylamide-SDS gels using a Mini Protean II Cell (Bio-Rad, Hercules, CA, USA). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Hybond-P; Amersham Biosciences, Buckinghamshire, U.K.) using a Mini Trans-blot Cell (Bio-Rad Laboratories, Hercules, CA, USA). Blot membranes were probed first with a rabbit polyclonal antiserum against PPV HC-Pro and then with a goat anti-rabbit antibody conjugated with horseradish peroxidase (Nordic Immunology, Tilburg, The Netherlands). Detection was carried out with the ECL system (Amersham Biosciences, Buckinghamshire, U.K.). The polyclonal antiserum against PPV HC-Pro was produced by immunization of rabbits with purified HC-Pro protein expressed in *E. coli* (Martínez-García, unpublished).

Detection of transiently expressed GUS protein. Plants were agroinfiltrated with pBI121 as described (Tenllado and Díaz-Ruíz, 2001). *Agrobacterium*-mediated transient expression of β -glucuronidase (GUS) was

determined two days after agroinfiltration, using the GUS-light chemiluminescence kit (Tropix Inc., Bedford, MA, USA), following the manufacturer's instructions. Light emission was quantified using a luminometer and the values obtained were compared to those from known quantities of purified β -glucuronidase (Roche Diagnostic, Indianapolis, USA). Agroinfiltrated tissue from transgenic plants was completely removed after GUS activity measurement, and then upper leaves were inoculated with PPV to assess PPV-resistance.

Extraction and analysis of RNA. Total RNA for viral RNA detection by either Northern blot or Reverse transcription (RT)-PCR, was prepared using the guanidine hydrochloride method (Logemann *et al.*, 1987). Samples from systemic or inoculated leaves were collected two weeks after virus inoculation. Northern blots were performed using 5 μ g of total RNA in 0.8% agarose formaldehyde gels (Sambrook *et al.*, 1989) and transferred to positively charged nylon membranes (Roche Diagnostic, Indianapolis, USA). Membranes were cross-linked in a Stratalinker 1800 (Stratagene, La Jolla, CA, USA) and hybridized overnight at 65°C with a digoxigenin (DIG)-labelled RNA probe (DIG RNA labelling kit, Roche Diagnostic Indianapolis, USA) complementary to the HC-Pro sequence. Detection was done with the chemiluminescent substrate CSPD (Roche Diagnostic, Indianapolis, USA).

RT-PCR was performed using AMV reverse transcriptase (Promega Corp., Madison, WI, USA) and 1 μ g of total RNA as template. The primers used amplify a portion of the CP gene of PPV. The upstream primer was 5' CTGCAGTCACGTCAGCAACAAC 3' (in italics nucleotides 8722 to 8738 of the PPV sequence (Laín *et al.*, 1989)). The downstream primer was 5' GTCGACATTGGATACTCCACTTG 3' (in italics nucleotides complementary to 9183 to 9199 of the PPV sequence).

For detection of HC-Pro transgene mRNA, total RNA was extracted with the RNeasy plant mini kit (Qiagen, Hilden, Germany) from 5 week-old plants one day before virus inoculation. For detection of transiently expressed GUS or 54-kDa-protein gene mRNAs, total RNA was extracted from agroinfiltrated leaves two days after agroinfiltration. Total RNA (20 μ g) was subjected to electrophoresis as above. Transgene mRNA and transiently expressed GUS mRNA were detected with a DIG-labelled RNA probe complementary to the GUS coding sequence. The 54-kDa-protein gene mRNA was detected with a complementary DIG-labelled RNA probe (Tenllado and Díaz-Ruiz, 2001). To confirm equal loading of RNA in each sample, blots were hybridized with a DIG-labelled RNA probe complementary to 18S ribosomal RNA (Ruiz-García *et al.*, 1997). Densitometry analyses of blots were performed in a calibrated densitometer GS-800 (Bio-Rad Laboratories, Hercules, CA, USA), using the Quantity One (Bio-Rad

Laboratories, Hercules, CA, USA) software. The intensities of the bands in each lane were normalized with that of the 18S ribosomal RNAs.

For siRNA analysis total RNA was extracted with Trizol (Invitrogen, San Diego, CA, USA) and low molecular weight (LMW) RNA was further purified using the DNA/RNA Midi kit (Qiagen, Hilden, Germany). Twenty μ g of LMW RNA were run in 15% polyacrylamide gels containing 7 M urea and 0.5xTBE. Gels were blotted onto positively charged nylon membranes (Roche Molecular Biochemicals, Indianapolis, USA) using a Trans-blot semi-dry transfer Cell (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were cross-linked in a Stratalinker 1800 (Stratagene, La Jolla, CA, USA) and hybridized overnight at 38°C in PerfectHyb Plus buffer (Sigma, St. Louis, USA), with a ³²P-labelled probe specific for the GUS sequence produced by random priming. Membranes were washed at 50°C twice in 2xSSC 0.2% SDS and twice in 0.1xSSC 0.2% SDS and subsequently exposed to film.

RESULTS

Transformation of *N. benthamiana* with the PPV HC-Pro gene. *N. benthamiana* plants were transformed with the construct pBI-HCT (Fig 1). This construct contains the entire PPV HC-Pro coding sequence supplemented with start and stop codons, placed between the CaMV 35S promoter and the GUS coding sequence of the binary vector pBI121 (Jefferson *et al.*, 1987). This construct is predicted to yield in plants an mRNA comprising the HC-Pro sequence followed by the GUS sequence, however only the HC-Pro, which is the first ORF in the mRNA, would be translatable. Additionally transgenic plants carrying the empty vector pBI121 were also generated to be used as controls. The transgenic status of regenerated plants was confirmed by PCR using specific primers (data not shown). Transgenic plants did not show any abnormal phenotype, being indistinguishable from non-transformed plants. Seven HC-Pro transgenic lines (HC1, HC2, HC3, HC13, HC15, HC18 and

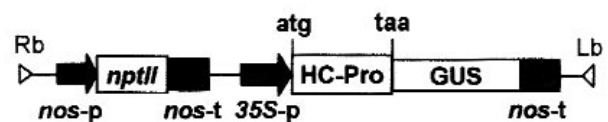


Fig. 1. Construct used in transformation of *N. benthamiana*. The T-DNA in pBI-HCT, flanked by the right (Rb) and left (Lb) borders, contains the kanamycin resistance *nptII* gene under the control of a *nos* promoter (*nos-p*) and terminator (*nos-t*), and the HC-Pro transgene, composed of the HC-Pro coding sequence including translational start and stop codons followed by the GUS gene sequence, under the control of a CaMV 35S promoter and *nos* terminator.

HC20) were used for subsequent experiments. Segregation of the kanamycin-resistance trait indicated that these HC-Pro transgenic lines contained between one and three transgene loci, as indicated in Table 1.

Expression of HC-Pro in different transgenic plants was analysed by western blotting. A band of the expected size (approx. 50 kDa) corresponding to HC-Pro was detectable in the HC lines tested. Dilutions of PPV-infected extracts were used as reference. In every case, accumulation of transgene-expressed HC-Pro was below the 1/200 dilution of PPV-infected plants (Fig. 2).

Resistance to PPV. HC-Pro transgenic plants from the R₁ generation were challenged with PPV 5.15. In addition, plants transformed with pBI121 and non-transgenic *N. benthamiana* plants were used as controls. For all experiments, we used a PPV 5.15 stock inocu-

lum that produced a mean of seven local lesions per leaf in the host *C. foetidum*. All the control plants inoculated with PPV 5.15 were infected, displaying symptoms of vein clearing in systemic leaves at 10-12 days post-inoculation and later symptoms of mosaic. Five of the seven HC-Pro transgenic lines tested (HC1, HC3, HC15, HC18 and HC20) were susceptible to PPV 5.15 infection, with all plants showing identical symptoms as control plants. In contrast, two HC-Pro lines (lines HC2 and HC13) showed resistance to PPV 5.15, in several different experiments, manifested as a high number of plants (approx. 69% and 88%, respectively) free of viral symptoms during their entire life (Table 1). The rest of the plants from these two lines were susceptible to PPV 5.15 showing the same kind of symptoms as control plants.

PPV RNA accumulation was tested in resistant and susceptible plants from lines HC2 and HC13 by Northern blotting. No viral RNA could be detected in RNA extracts from systemic leaves of resistant plants, while susceptible plants accumulated PPV RNA at the same level as control infected plants (Fig. 3A). We also checked virus accumulation in the inoculated leaves. No viral RNA was detected from inoculated leaves of resistant plants (Fig. 3B), indicating that PPV 5.15 proliferation was blocked soon after entrance of the virus into the plant cells. Virus accumulation was investigated further in line HC2 using RT-PCR as a more sensitive technique. A PPV-specific product corresponding to a portion of the CP gene could be amplified from RNA extracts derived from susceptible transgenic plants and control infected plants using 1 µg of total RNA as template (Fig. 3C). This PCR product could be amplified using as little as 1.6 ng of total RNA extracts derived

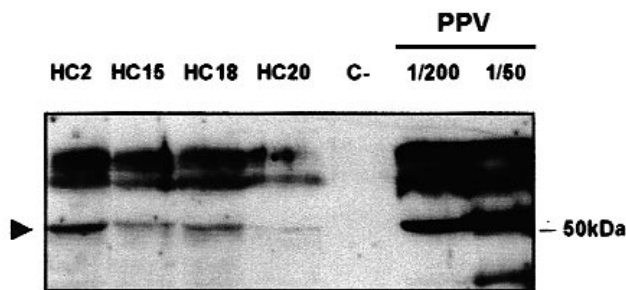


Fig. 2. Detection of HC-Pro in different transgenic lines. Proteins were extracted from R₁ generation plants. Plants transformed with the vector pBI121 were used as negative control (C-). As positive control, an extract from a PPV-infected plant was diluted as indicated in healthy non-transformed plant extract. The arrow indicates the band corresponding to the HC-Pro protein.

Table 1. HC-Pro transgenic lines challenged with *Plum pox virus* (PPV) 5.15 isolate.

Lines	Resistance to PPV 5.15	(Km ^r /Km ^s) ^b	Segregation ratio ^c	Loci no. ^d
<i>N. benthamiana</i> ^e	0/4, 0/5 ^a	-	-	-
pBI121 ^f	0/10, 0/10, 0/8, 0/7	nt ^g	nt	nt
HC1	0/10	156/57	3:1	1
HC2	7/10, 9/10, 6/10, 11/18	477/13	63:1	3
HC3	0/10	145/36	3:1	1
HC13	7/7, 8/10	125/8	15:1	2
HC15	0/18	242/66	3:1	1
HC18	0/8	218/83	3:1	1
HC20	0/8	236/71	3:1	1

^aPlants inoculated with PPV 5.15, in different experiments. Values represent number of PPV-resistant plants/number of total inoculated plants.

^b Segregation of kanamycin resistance in the R₁ progeny. (Km^r) number of kanamycin-resistant seedlings; (Km^s) number of kanamycin-sensitive seedlings.

^cBest fitting kanamycin-segregation ratios.

^dNumber of independent loci estimated by kanamycin resistance segregation.

^eNon-transgenic *N. benthamiana* plants.

^fPlants transformed with the empty vector pBI121.

^gNot tested.

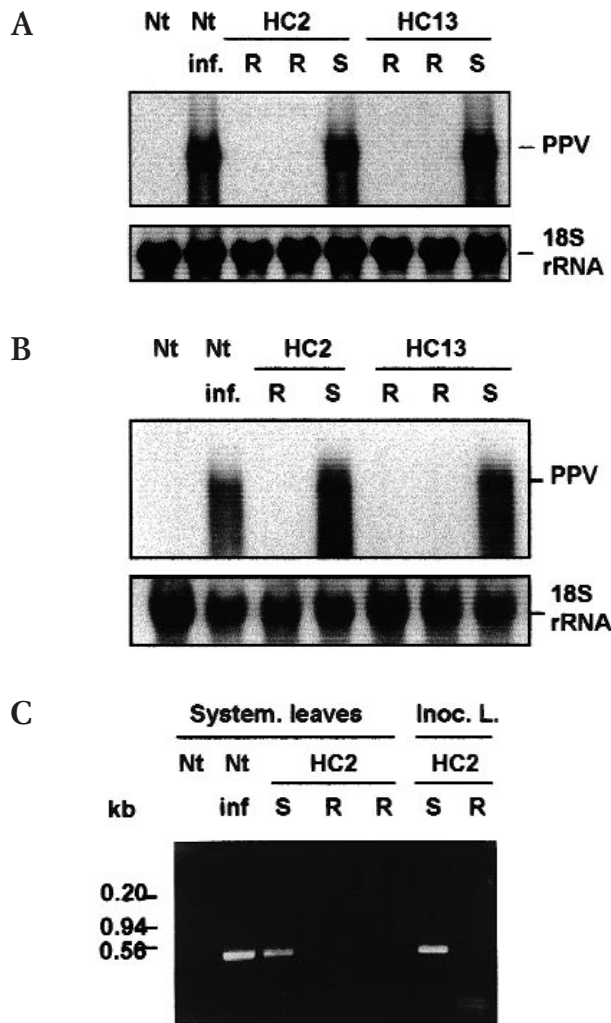


Fig. 3. Detection of PPV RNA in HC2 and HC13 transgenic lines. Total RNA was extracted two weeks after PPV inoculation from transgenic plants displaying a resistant (R) or susceptible (S) phenotype to PPV infection. Non-transgenic plants (Nt) infected with PPV 5.15 (inf.) or non-infected were used as controls. **A)** Accumulation of viral RNA in systemic leaves. **B)** Accumulation of viral RNA in inoculated leaves. Equal loading of RNA samples was assessed using a DIG-labelled RNA probe complementary to the 18S ribosomal RNA (lower panel). **C)** Detection of PPV by RT-PCR. Primers used amplify a portion of the CP coding sequence.

from infected plants (data not shown). In contrast, no PPV-derived PCR product could be detected in RNA extracts from either systemic or inoculated leaves of HC2 resistant plants using 1 µg of total RNA.

Line HC2 was challenged with a more infectious PPV 5.15 inoculum. For this purpose we used a fresh PPV 5.15 inoculum that produced a mean of 20 local lesions per leaf in *C. foetidum*. Eight out of nine HC2 plants inoculated remained free of symptoms, while only one plant became infected, displaying the same symptoms of vein-clearing and mosaic as control plants. This high percentage of resistant plants, very similar to that obtained using the stock inoculum, suggests that the resistance is

not greatly affected by the strength of the inoculum used.

We tested the specificity of the resistance mechanism by challenging line HC2 with three different potyviruses. We used PPV 3.3, PVY and TEV, which share 99%, 55% and 55% nucleotide sequence homology with the PPV HC-Pro transgene, respectively. Ten HC2 plants and six control plants were inoculated with each virus. All the control plants were infected with each of the three different viruses. Interestingly, all plants from line HC2 were infected with either PVY or TEV. However, only three out of ten plants inoculated were infected with PPV 3.3. All together these data indicate that resistance to PPV is caused by a strong, homology-dependent mechanism.

PTGS of the HC-Pro transgene and resistance to PPV. Virus resistance in transgenic plants carrying virus-derived transgenes is frequently caused by PTGS of the transgene. To test this possibility we investigated transgene mRNA accumulation and transgene-derived siRNA accumulation in plants from line HC2. For transgene mRNA analysis, tissue samples were collected and plants were subsequently inoculated with PPV in order to determine their resistance phenotypes. Transgene mRNA was detected in all the HC2 plants examined (Fig. 4A). However, transgene mRNA accumulation was greatly reduced in PPV-resistant plants when compared to the level observed in susceptible ones. This difference ranged from approximately 2 to 8-fold as determined by densitometry analysis. In accordance with this, transgene-derived siRNAs were detected only in resistant HC2 plants (Fig. 4B). This observation supports the conclusion that the resistance to PPV displayed by a portion of HC2 transgenic plants is caused by PTGS of the HC-Pro transgene.

Trans-inactivation of a transiently expressed GUS gene homologous to the transgene. We tested the capability of PTGS induced by the HC-Pro transgene to target homologous RNA species other than PPV RNA. For this purpose we tested the targeting or trans-inactivation (English *et al.*, 1997; Llave *et al.*, 2000) of a transiently expressed GUS gene. As the HC-Pro transgene comprises the HC-Pro sequence followed by a non-translatable GUS sequence, we expected that PTGS induced by the HC-Pro transgene would also target a transiently expressed GUS gene. Transgenic plants from lines HC2, HC3, HC15 and HC18 were infiltrated with *Agrobacterium* carrying pBI121 that contains a functional GUS gene. After two days GUS activity was measured. All of the plants from lines HC3, HC15 and HC18 showed high levels of GUS activity. In contrast, 19 out of 25 plants from line HC2 displayed reduced levels (20-fold) of GUS activity compared to the rest of the plants in this line that showed high levels (Fig. 5A). Later on, inoculation of these plants with PPV showed

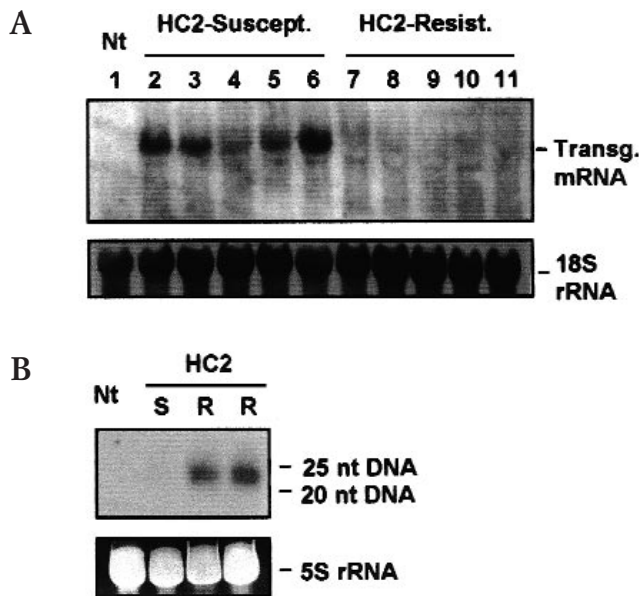


Fig. 4. Detection of HC-Pro transgene mRNA and siRNAs in HC2 plants. **A**) For mRNA detection, samples were taken from 5 week-old plants, one day before PPV inoculation. Total RNA was extracted from either HC2 plants susceptible (lanes 2-6) or resistant (lanes 7-11) to PPV, or from non-transgenic plants (Nt, lane 1). Transgene mRNA was detected using a DIG-labelled RNA probe complementary to the GUS portion of the transgene (upper panel). Equal loading of RNA samples was assessed using a DIG-labelled RNA probe complementary to the 18S ribosomal RNA (lower panel). **B**) siRNAs derived from the transgene were assayed in PPV-susceptible (S) or PPV-resistant (R) HC2 plants using a ³²P-labelled probe specific for the GUS sequence. 20 and 25 nucleotides DNA oligonucleotides were used as size markers. An ethidium bromide staining of the gel before transferring is shown at the bottom as loading control.

a strict correlation between decreased GUS activity and virus resistance; all of the 19 plants with decreased GUS activity were resistant to PPV, suggesting that trans-inactivation of the transiently expressed GUS gene and resistance to PPV were both caused by the same transgene-induced PTGS.

Trans-inactivation of the transiently expressed GUS gene was investigated further at the RNA level. *Agrobacterium* cultures carrying either pBI121 or pBI/54-kDa, were mixed and coinfiltrated. The construct pBI/54-kDa expresses a sequence derived from *Pepper mild mottle virus* (PMMoV) with no significant homology with the PPV HC-Pro transgene, and was used as internal control of the assay. PPV-resistant plants from line HC2 accumulated approximately 10-fold less GUS mRNA than PPV-susceptible HC2 and HC3 plants (Fig. 5B). In contrast, accumulation of the 54-kDa-protein gene mRNA was similar in all the infiltrated plants, confirming that the trans-inactivation of the GUS gene is sequence-specific and probably caused by PTGS.

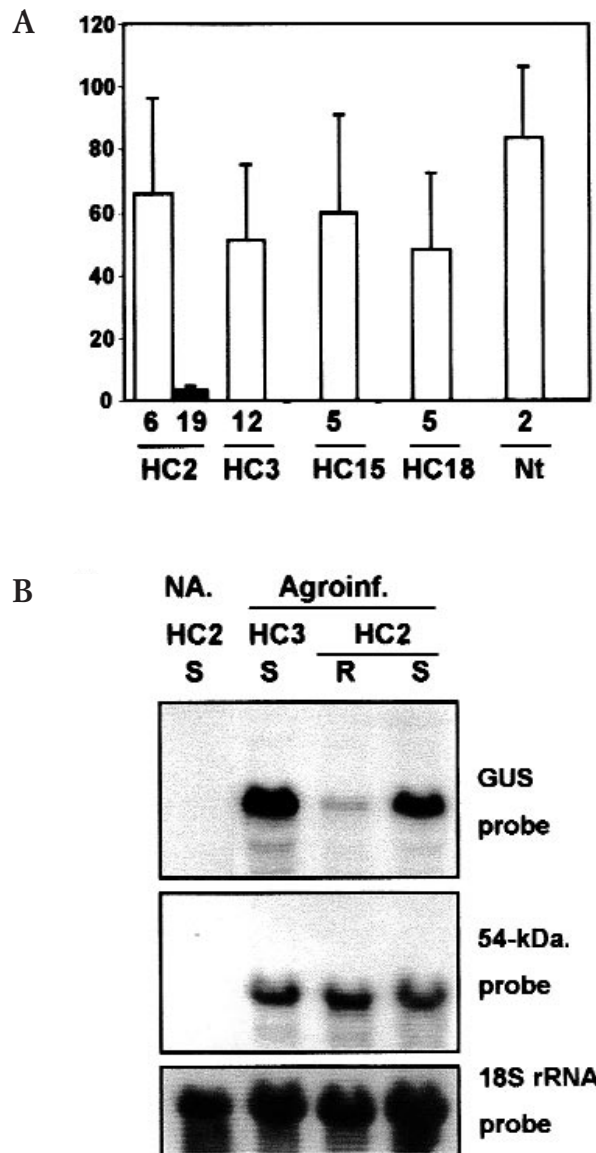


Fig. 5. Trans-inactivation of a transiently expressed GUS gene. **A**) Levels of β -glucuronidase (GUS) activity in plants from different HC-Pro lines resistant (black bar) or susceptible (white bars) to PPV. Leaves of HC-Pro plants were agroinfiltrated with pBI121, which carries a functional GUS gene. GUS activity was measured two days post-agroinfiltration and the values obtained were compared to those of known quantities of purified β -glucuronidase. The number of plants tested for each HC-Pro line is indicated below the corresponding bar. Bars represent mean values, expressed as nanograms of β -glucuronidase per milligram of tissue. Lines above bars represent the standard deviation of the mean values. **B**) Detection of mRNA from transiently expressed GUS or 54-kDa-protein genes in HC-Pro plants resistant (R) or susceptible (S) to PPV. Leaves were infiltrated with a mix of *Agrobacterium* cultures carrying either pBI121 or pBI/54-kDa. After two days, total RNA was extracted from agroinfiltrated and non-agroinfiltrated (NA) leaves. Either GUS or 54-kDa-protein gene mRNAs were detected using specific DIG-labelled RNA probes. Equal loading of RNA samples was assessed using a DIG-labelled RNA probe complementary to the 18S ribosomal RNA.

Suppression of PTGS by PVY infection. Infection of plants containing a post-transcriptionally silenced transgene with certain potyviruses, was shown to suppress PTGS of the transgene (Brigneti *et al.*, 1998; Savenkov and Valkonen, 2001; Simón-Mateo *et al.*, 2003). Using this approach, we tested suppression of PTGS in our HC-Pro transgenic plants by PVY infection. To ensure that silenced HC-Pro plants were used, HC2 plants that showed trans-inactivation of the transiently expressed GUS gene were selected for PVY inoculation. A strong increase in HC-Pro transgene mRNA accumulation in these HC2 plants was observed after PVY infection (Fig. 6). In contrast, no significant increase in transgene mRNA accumulation was observed in non-silenced HC3 plants after infection with PVY (using 18S rRNA signal as reference). Moreover, no significant increase in transgene mRNA accumulation was observed in upper leaves of non-inoculated HC2 plants, indicating that the increase in transgene mRNA accumulation observed in PVY-infected HC2 plants was specifically caused by PVY infection and not by developmental changes in transgene expression. Therefore the data indicate that PVY HC-Pro produced during infection can suppress PTGS of the PPV HC-Pro transgene.

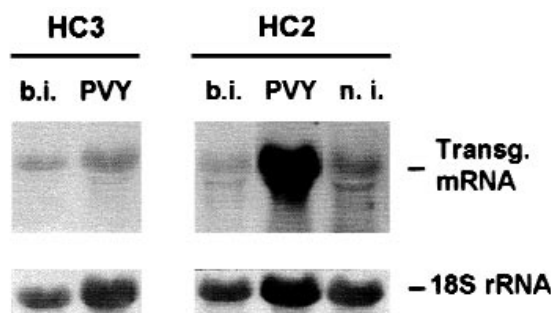


Fig. 6. Detection of transgene mRNA in HC-Pro plants before and after infection with PVY. Total RNA was extracted from leaf tissue one day before inoculation with PVY (b.i.) and from systemically PVY infected leaves (PVY) or equivalent leaves of non-inoculated plants (n.i.). HC-Pro transgene mRNA was detected using a specific DIG-labelled RNA probe. Equal loading of RNA samples was assessed using a DIG-labelled RNA probe complementary to the 18S ribosomal RNA.

DISCUSSION

Our results show a high level of resistance to PPV in some of the *N. benthamiana* plants transformed with a translatable version of the PPV HC-Pro gene. Resistance was also effective against a PPV strain very similar (99%) to that from which the transgene originated. In contrast, resistance was not effective against PVY and TEV, which share only 55% sequence homology to the transgene, a finding that suggests a homology-dependant

resistance mechanism based on PTGS. Resistance to PPV correlated with a decreased accumulation of transgene mRNA and the presence of siRNAs derived from the transgene, strongly suggesting that resistance to PPV is caused by PTGS of the HC-Pro transgene. Silencing of the HC-Pro transgene could also trans-inactivate a transiently expressed GUS gene encoded by pBI121 as it shares sequence identity with the 3' portion of the transgene, indicating that the transgene-induced PTGS can target either the GUS mRNA or the PPV viral RNA. Transient expression of genes by agroinfiltration is capable of inducing silencing of stably integrated homologous genes in some systems (Voinnet *et al.*, 1998). To minimize the possibility that silencing is induced by transient expression of pBI121, GUS activity was measured soon after agroinfiltration and the infiltrated leaves completely removed after that. The low accumulation of GUS protein and mRNA observed at that early stage suggests that silencing was pre-established by the HC-Pro transgene and was not a result of induction by transient expression of pBI121.

Resistance to PPV appears in lines containing several transgene inserts (three inserts in HC2 and two in HC13, as estimated by the kanamycin resistance segregation), which is a common feature in post-transcriptionally silenced plants (Smith *et al.*, 1994; Goodwin *et al.*, 1996). The proportion of PPV-resistant R_1 plants (69% and 88% in HC2 and HC13, respectively) is close to 75%, which corresponds in theory to the segregation ratio of a single gene in hemizygous state. This suggests that the presence or absence of a particular single insert of the transgene may be conditioning silencing and therefore virus resistance. Some features of this particular insert, like insertion of two transgene copies in an inverted repeat configuration or insertion in front of an endogenous promoter, may promote initiation of PTGS (Sijen and Kooter, 2000). Additionally, inclusion of the GUS sequence 3' to the HC-Pro coding sequence, might have positive effects in induction of PTGS as seen in previous works (Pang *et al.*, 1997) with the addition of heterologous sequences to the 3' end of the viral transgene.

The HC-Pro of potyviruses has been shown to suppress PTGS in several systems (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau and Carrington, 1998). This suppression was shown to act at the maintenance step as HC-Pro can interfere with silencing in cells in which PTGS is already established (Llave *et al.*, 2000). HC-Pro also affects accumulation of short-interfering RNAs (siRNA), which are characteristic of PTGS, however it does not inhibit the production of a systemic silencing signal (Mallory *et al.*, 2001). HC-Pro can efficiently suppress PTGS promoted by weak inducers, such as some transgene mRNAs or RNAs with limited amounts of dsRNA. However, it can only partially suppress PTGS promoted by strong inducers, such as transgenes producing long segments of dsRNA and replicat-

ing RNA viruses (Johansen and Carrington, 2001). Therefore, this silencing suppression process is likely to be a balance between suppression mediated by HC-Pro and induction of silencing by the PTGS initiator molecule, rather than a dominant suppression mediated by HC-Pro capable of overcoming any induction of silencing. In this regard, many transgenic systems expressing transgenes derived from potyviruses show recovery from virus infection, characterized by a progressive exclusion of the virus from newly developing tissues by a mechanism that resembles PTGS, despite the fact that HC-Pro is being produced from the virus (Marathe *et al.*, 2000; Voinnet, 2001).

Two previous papers have reported PTGS related phenomena in plants transformed with the HC-Pro of different potyviruses (Mlotshwa *et al.*, 2002; Savenkov and Valkonen, 2002). Only one of the lines described by Mlotshwa *et al.* (2002) showed spontaneous PTGS of the HC-Pro transgene. The other lines reported were not silenced and accumulated high amounts of HC-Pro. After infection with the homologous virus plants recovered, showing no symptoms and silencing of the HC-Pro transgene regardless of the presence of HC-Pro from the transgene and the virus. However, silencing in these systems seemed to be unstable, in contrast to other systems showing recovery (Lindbo *et al.*, 1993; Guo and García, 1997; Marathe *et al.*, 2000; Voinnet, 2001). In one case (Savenkov and Valkonen, 2002), recovered leaves were not completely resistant to a second inoculation with the virus. In the other case (Mlotshwa *et al.*, 2002), viral symptoms reappeared later in the recovered plants. These data suggest that the strong induction of PTGS promoted by the viral infection is capable of overcoming suppression of PTGS mediated by HC-Pro. However, after recovery PTGS could not be maintained stably in these plants, suggesting that the HC-Pro produced from the transgene can interfere with PTGS when the amount of silencing-inducer virus is reduced.

The aim of our study was to test if an HC-Pro-encoding transgene can be spontaneously silenced in plants causing resistance to the homologous virus or, alternatively, if silencing is suppressed by the transgene-expressed HC-Pro. Our results showed complete resistance to PPV in some of the HC-Pro transgenic plants, which is consistent with a PTGS process induced by the HC-Pro transgene that targets PPV viral RNA. In favour of this, resistant plants showed reduced levels of transgene mRNA and accumulation of siRNAs prior to PPV infection, which confirms the silencing of the HC-Pro transgene. These results suggest that the HC-Pro expressed from the transgene does not interfere with PTGS, at least in those lines showing silencing. Previous results showed that transient expression of pBI-HCT, the construct used for plant transformation, could interfere with silencing promoted by an inverted repeat construct and partially suppressed resistance against a virus bear-

ing sequence identity with the construct (Tenllado *et al.*, 2003). In the present study, expression of HC-Pro by stably integrated transgenes is probably lower than *Agrobacterium*-mediated transient expression. However, this transgene-expressed HC-Pro might be enough to interfere with PTGS, especially that elicited by weak inducers. Establishment of PTGS in HC-Pro plants implies that PTGS is elicited by relatively strong inducers of PTGS or alternatively that PTGS is achieved very soon during development, decreasing transgene mRNA level and therefore preventing HC-Pro accumulation. On the other hand, higher amounts of HC-Pro produced by PVY infection could efficiently suppress PTGS of the transgene, supporting the idea that the outcome depends on the relative amounts of PTGS elicitor and suppressor.

Suppression of PTGS seems necessary for viruses to avoid plant defences that limit their spreading and accumulation (Kasschau and Carrington, 2001). However, a very strong suppression of PTGS-based plant defences or too high a virus accumulation, could be detrimental for the host plant and hence for virus perpetuation. The fact that some viral suppressors of PTGS can interfere with micro-RNA mediated developmental regulation (Kasschau *et al.*, 2003; Chapman *et al.*, 2004; Dunoyer *et al.*, 2004; Silhavy and Burgyan, 2004) also suggests that a very strong suppression of PTGS could be too detrimental for the host plant. It has been reported that in *Beet western yellows virus* (BWYV) the PTGS suppressor gene P0 is poorly translated due to a suboptimal translation initiation codon. Moreover there is a selection pressure that favours this suboptimal initiation codon avoiding overexpression of the PTGS suppressor gene P0 (Pfeffer *et al.*, 2002). Selective pressures might have favoured a balance between plant defences based on PTGS and virus counter-defences based on suppression of PTGS.

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