

## Silencing of a viral RNA silencing suppressor in transgenic plants

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### Abstract

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High expression levels of the helper component proteinase (HC<sup>pro</sup>), a known virus suppressor of RNA silencing, were attained in *Nicotiana benthamiana* transformed with the HC<sup>pro</sup> cistron of *Potato virus A* (PVA, genus *Potyvirus*). No spontaneous silencing of the HC<sup>pro</sup> transgene was observed, in contrast to the PVA coat protein (CP)-encoding transgene in other transgenic lines. HC<sup>pro</sup>-transgenic plants were initially susceptible to PVA and were systemically infected by 14 days post-inoculation (p.i.) but, 1 to 2 weeks later, the new expanding leaves at positions +6 and +7 above the inoculated leaf showed a peculiar recovery phenotype. Leaf tips (the oldest part of the leaf) were chlorotic and contained high titres of PVA, whereas the rest of the leaf was symptomless and contained greatly reduced or non-detectable levels of viral RNA, CP and transgene mRNA. The spatial recovery phenotype suggests that RNA silencing is initiated in close proximity to meristematic tissues. Leaves at position +8 and higher were symptomless and virus-free but not completely resistant to mechanical inoculation with PVA. However, they were not infected with the virus systemically

transported from the lower infected leaves, suggesting a vascular tissue-based resistance mechanism. Recovery of the HC<sup>pro</sup>-transgenic plants from infection with different PVA isolates was dependent on the level of sequence homology with the transgene. Methylation of the HC<sup>pro</sup> transgene followed recovery. These data show that the transgene mRNA for a silencing suppressor can be silenced by a presumably 'strong' silencing inducer (replicating homologous virus).

## Introduction

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RNA silencing is a host defence mechanism targeted against invasive or mobile RNA elements, such as viruses or transposable retro-elements, leading to sequence-specific RNA degradation (reviewed by Baulcombe, 1999; Chicas & Macino, 2001; Matzke *et al.*, 2001; Vance & Vaucheret, 2001; Waterhouse *et al.*, 2001). It is highly conserved in plants and animals and known as post-transcriptional gene silencing (PTGS) in plants. RNA silencing was discovered in transgenic plants expressing transgenes from strong promoters, e.g., the 35S promoter of *Cauliflower mosaic virus* (CaMV), and which produce stable (polyadenylated) mRNAs. In such plants, the highly expressed mRNA can be inactivated in a sequence-specific manner in the cytoplasm (van der Krol *et al.*, 1990; Napoli *et al.*, 1990).

Plant viruses encode proteins able to suppress RNA silencing, probably to facilitate virus replication and systemic movement (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau & Carrington, 1998; Mallory *et al.*, 2001; Voinnet *et al.*, 1999, 2000). Virus silencing suppressors have been valuable tools for dissecting RNA silencing pathway(s), consisting of initiation, maintenance and propagation (signalling) phases (reviewed by Carrington *et al.*, 2001). Initiation of RNA silencing is dependent on the presence of dsRNA and results in establishment of a silent state for targeted genes or RNAs. The maintenance phase is associated with homology-dependent transgene methylation of unknown significance. The propagation phase of silencing is manifested by the spread of silencing from the initially silenced cells to non-silenced cells, tissues and different parts of the plant. The putative signal is transported systemically similarly to viruses and photoassimilates (Voinnet & Baulcombe, 1997; Citovsky & Zambryski, 1999). Short (21–26 nt) sense and antisense RNAs complementary to the targeted RNA (Hamilton & Baulcombe, 1999; Papaefthimiou *et al.*, 2001) may function as the systemic silencing signal, perhaps as a complex with a nuclease (Hammond *et al.*, 2000).

Much of the mechanism of RNA silencing is still unknown. No model seems to accommodate all phenomena known to lead to the silent state. Some models predict a threshold level of RNA accumulation to be required for transgene-induced silencing of homologous viral

RNAs. Accordingly, an abundance of viral RNA exceeding a threshold level would trigger RNA silencing, consistent with silencing triggered by highly transcribed single-copy loci, and enable plant recovery from virus infection (Lindbo & Dougherty, 1992; Lindbo *et al.*, 1993; Mueller *et al.*, 1995). The term recovery implies here that transgenic plants expressing virus-derived transgenes are initially susceptible to the homologous viruses and become systemically infected and show typical symptoms, but the new leaves, which develop later, are symptomless, virus-free and resistant to new infections with the same virus (Lindbo & Dougherty, 1992). A threshold sequence similarity between the transgene and the infecting virus is also needed for initiation of silencing. The threshold sequence similarity seems to vary depending on viruses and viral genes used for plant transformation (Longstaff *et al.*, 1993; Pang *et al.*, 1993; Mueller *et al.*, 1995; Taliansky *et al.*, 1998). In transgenic pea plants expressing the replicase gene (NIB) of *Pea seed-borne mosaic virus* (PSbMV), 89 % sequence identity was required for activation of resistance (Jones *et al.*, 1998a).

The helper component proteinase (HC<sup>pro</sup>) of potyviruses (genus *Potyvirus*) can reverse the maintenance phase of RNA silencing and re-establish transgene expression (Brigneti *et al.*, 1998). However, it is not known whether an HC<sup>pro</sup>-encoding transgene can be spontaneously silenced in plants in spite of HC<sup>pro</sup> expression and whether such transgenic plants are susceptible to infection with the homologous virus. Therefore, the aim of this study was to test the interaction between an HC<sup>pro</sup>-expressing transgene and virus isolates carrying sequences identical or highly homologous to the transgene mRNA. The results indicate that plants expressing HC<sup>pro</sup> are initially susceptible to infection with the homologous viruses but later exhibit strong silencing of both the transgene and the homologous virus, revealed as a recovery from infection. The initial stages of recovery were characterized by a peculiar 'lanceolate leaf tip' (LLT) phenotype, which suggests that initiation of silencing occurs in close proximity to meristematic tissues. Furthermore, our results indicate that recovery is dependent on the sequence similarity between the virus and the transgene.

## Methods

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**Transformation of *Nicotiana benthamiana* and analysis of the transgenic plants.** The transgenic plant materials and conditions used for plant culture have been described (Savenkov & Valkonen, 2001a). PCR was carried out as described by Wang *et al.* (1993) to check the T1 progeny for possible non-transgenic segregants, which were discarded.

**Potato virus A (PVA) isolates, inoculation and virus detection.** PVA isolates used in this study have been described (Rajamäki *et al.*, 1998; Kekarainen *et al.*, 1999). Concentrations of PVA and HC<sup>pro</sup> in leaves were estimated by ELISA using rabbit polyclonal antibodies (courtesy of F. Rabenstein, BAFZ, Aschersleben, Germany), as described previously (Savenkov & Valkonen, 2001a).

**RNA extractions and RNA gel blot analysis.** Total RNA was extracted from ~100 mg of plant tissue (Verwoerd *et al.*, 1989). Total RNA (10–30 µg) was electrophoresed through 2.2 M formaldehyde–agarose gels and blotted onto Hybond-N<sup>+</sup> membranes (Amersham). PCR with HC<sup>pro</sup> gene-specific primers was used to generate [<sup>32</sup>P]dCTP-labelled deoxyriboprobes. The primer sequences were (5' forward primer) 5' TCACATCAGAGGGTATTAAATC 3' and (3' primer) 5' GAATACAGTGGAGTGCCATCAT 3'. Filters were hybridized in hybridization buffer (5× Denhardt's solution, 6× SSC, 0.5 % SDS and 0.5 mg/ml boiled herring sperm DNA) overnight at 65 °C and washed twice with a final solution of 2× SSC (1× SSC contains 0.15 M NaCl and 0.015 M sodium citrate) for 10 min each and once with 2× SSC supplemented with 0.1 % SDS for 30 min at 70 °C. The filters were exposed to PhosphorImager screens for image detection (Molecular Dynamics). RNA size markers (GibcoBRL) were used to determine the size of the transgene mRNA and genomic viral RNA.

**Methylation analyses.** Total genomic DNA was isolated from individual plants of the experimental line ab34 using a DNAeasy Plant Mini kit (Qiagen). Equal amounts of total DNA (~100 ng) were digested for 14 h at 37 °C with 20 units of *Cla*I or *Not*I restriction endonucleases (Fermentas). Primers flanking the *Cla*I or *Not*I restriction sites were designed. The sequence of the 3' reverse primer (R1) used for all PCR analyses was 5' GAATACAGTGGAGTGCCATCAT 3'. The sequences of the three forward primers used in our experiments were: (F1) 5' CTGGCGAGGATTCAATCGAAC 3'; (F2) 5' TCACATCAGAGGGTATTAAATC 3'; and (F3) 5' CAAGACCCTTCCTCTATATAAG 3'. The F1/R1 primer pair amplified a 0.89 kb fragment, indicative of methylation at the *Cla*I site, when *Cla*I-digested samples were used for PCR amplification (see Fig. 6). This same primer pair served for amplification of the short control fragment from *Not*I-digested samples. The F2/R1 primer pair amplified a 0.60 kb control band for *Cla*I-digested samples. The F3/R1 primer pair amplified a 1.13 kb fragment, indicative of methylation at the *Not*I site, when *Not*I-digested samples were used for PCR amplification.

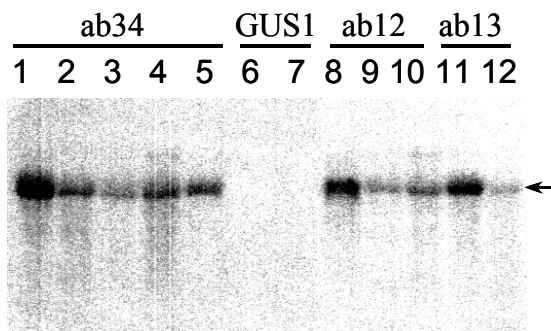
The samples of total DNA (either undigested or digested with *Cla*I or *Not*I) were

subjected to PCR amplification using the appropriate primer pair. At least two plants were tested at each time-point and for each PVA isolate. The PCR conditions were as follows: the hot-start represented a 4 min denaturing step at 94 °C, followed by 35 cycles consisting of denaturation (1 min at 94 °C), primer annealing (1 min at 58 °C) and strand elongation (1 min at 72 °C). PCR products were separated by size in 1 % agarose gels containing ethidium bromide.

## Results

### Main properties of the transgenic *N. benthamiana* plants

In a previous study, several transgenic lines of *N. benthamiana* (ab12, -13 and -34) expressing the PVA HC<sup>pro</sup> were produced, characterized and self-pollinated for production of T1 progeny (Savenkov & Valkonen, 2001a). The transgenic lines and T1 progeny plants expressed high levels of HC<sup>pro</sup> mRNA, as shown by Northern blot analysis (Fig. 1), and HC<sup>pro</sup>, as measured by ELISA using antibodies to HC<sup>pro</sup> (Savenkov & Valkonen, 2001a). The T1 progeny of line ab34 was chosen for this study and the seeds grown for experiments. Controls included the T1 progeny of a line of *N. benthamiana* (ab10) transformed with the PVA coat protein (CP) gene and the T1 progeny of another transgenic line (GUS1) expressing the GUS marker gene. These transgenic lines and their transgene expression have been described previously (Savenkov & Valkonen, 2001a, b). The main characteristics of the transgene constructs and the transgenic lines are summarized in Table 1.



**Fig. 1.** Accumulation of steady-state levels of transgene mRNA (arrow) in the T1 progeny plants (two leaves stage) of three *N. benthamiana* lines transformed with the PVA 5' UTR+HC<sup>pro</sup> construct (see Fig. 6). Each lane represents RNA from an individual T1 progeny plant. Lanes: 1–5, line ab34; 8–10, line ab12; 11 and 12, line ab13. RNA from T1 progeny plants (lanes 6 and 7) of a transgenic line (GUS1) expressing the GUS marker gene were included as negative controls. Total RNA was

extracted from young leaves and 10 µg RNA from each sample was subjected to Northern blot analysis using <sup>32</sup>P-labelled PVA HC<sup>pro</sup> cDNA as a probe.

**Table 1.** Main characteristics of the *N. benthamiana* plants transformed with the HC<sup>pro</sup>- or CP-encoding sequences of PVA

Transgenic line	Gene	Binary construct			Transgene expression detected by		
		35S promoter	Translational enhancer*	Plant intron	RT-PCR	Northern blot	Protein assay†
ab34	HC <sup>pro</sup>	Yes	Yes	No	Yes	Yes	Yes
ab10	CP	Yes	Yes	No	Yes	No	No
GUS1	GUS	Yes	No	Yes	NT	NT	Yes

\* PVA leader (5' UTR) sequence.

† In case of the GUS1 line, expression was tested by  $\beta$ -glucuronidase histochemical assay.

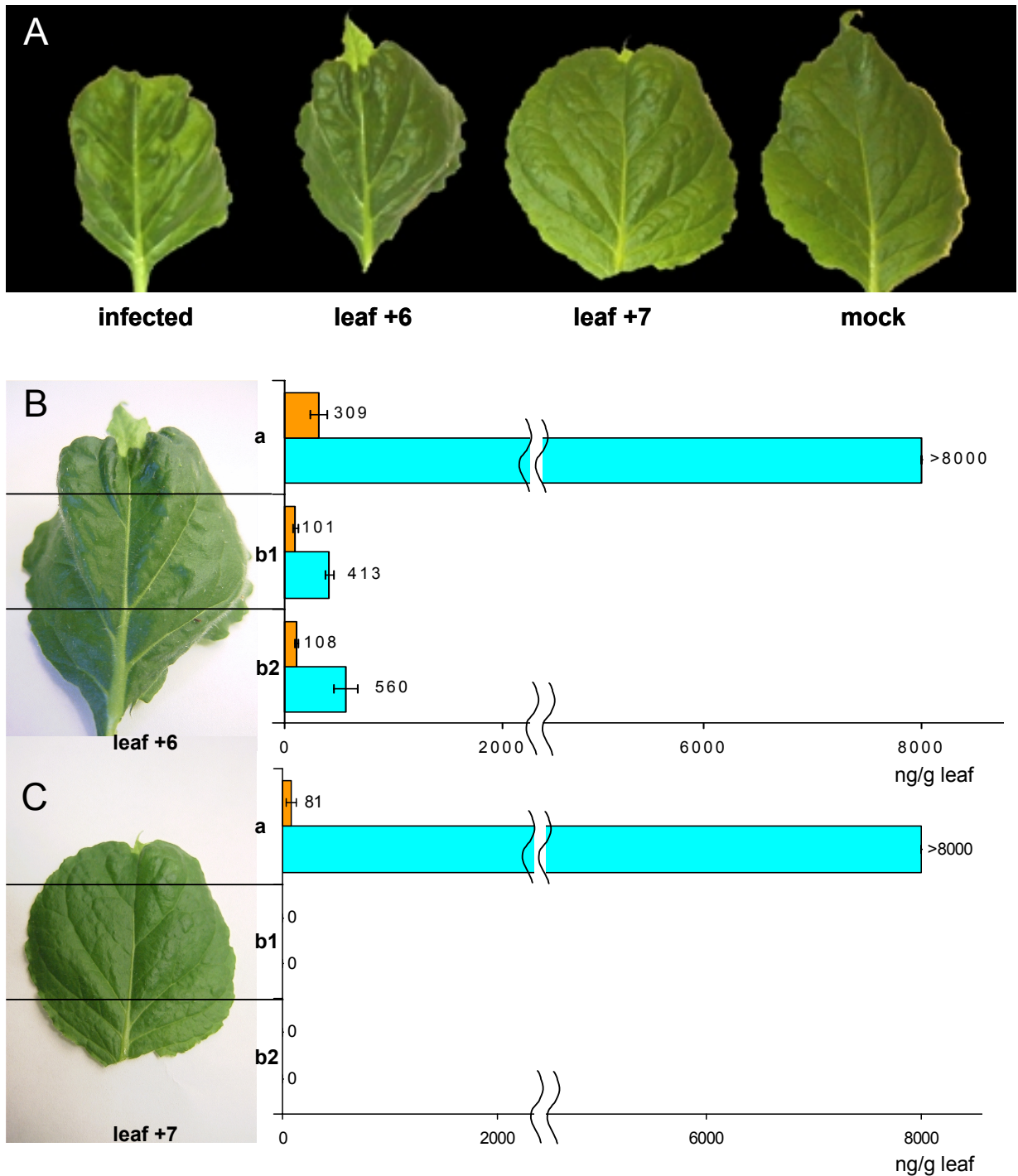
NT, Not tested.

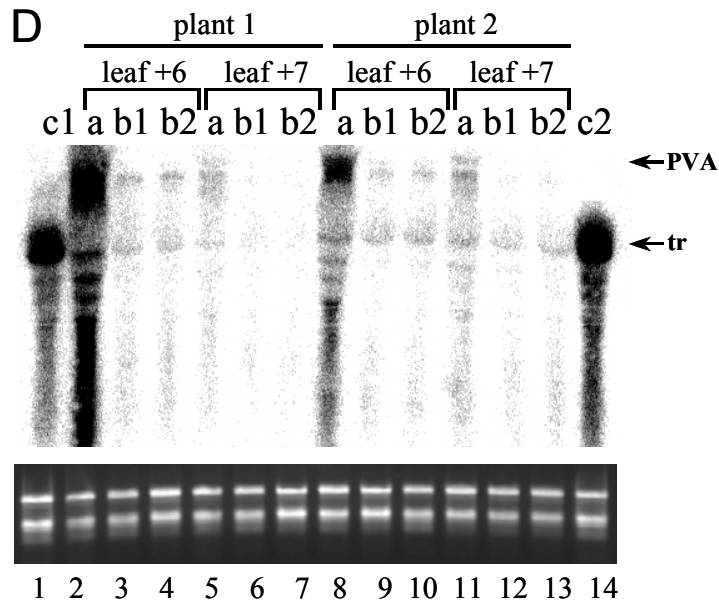
### Responses to challenge inoculation with the homologous PVA isolate B11

Responses of the HC<sup>pro</sup>-transgenic and wt *N. benthamiana* plants to PVA infection were initially tested by mechanical inoculation with PVA isolate B11, the isolate from which the HC<sup>pro</sup> and CP transgenes were derived. Similar severe symptoms of mosaic and malformation of leaves were observed in the HC<sup>pro</sup>-transgenic and wt *N. benthamiana* plants at 14 days p.i. Virus titres were also similar, as estimated by ELISA, in the systemically infected leaves (HC<sup>pro</sup>-transgenic plants: 39  $\mu\text{g/g}$  leaf,  $n=26$ ; wt control plants 37.7  $\mu\text{g/g}$  leaf,  $n=18$ ). Thus, HC<sup>pro</sup> expression in the transgenic plants had no detectable effect on PVA accumulation and disease phenotype at an early stage of systemic infection.

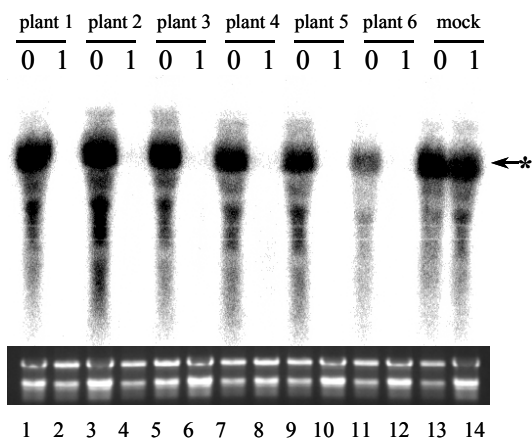
However, at 21–28 days p.i. with PVA isolate B11, a peculiar phenotype was observed in the expanding sixth (position +6) and seventh (position +7) leaf above the inoculated leaf (position 0) in the HC<sup>pro</sup>-transgenic plants. These leaves had an elongated, narrow leaf tip that was chlorotic (yellow) (Fig. 2A). All new leaves above those with the LLT phenotype developed without symptoms. The inoculated leaves (position 0) and all leaves above the inoculated leaves (positions 1–10) from several HC<sup>pro</sup>-transgenic plants and PVA-infected control plants were tested for PVA by ELISA at 21 days p.i. Only the inoculated leaves and the upper leaves at positions 1–5 were PVA-infected for the HC<sup>pro</sup>-transgenic plants, whereas the symptomless leaves at positions 8–10 were virus-free, as determined by ELISA and Northern blot analysis (Fig. 3). In contrast, all leaves at positions 0–10 were PVA-infected in the GUS1 and wt control

plants, showed severe symptoms and no LLT phenotype was observed. The response of all HC<sup>pro</sup>-transgenic lines (ab12, -13 and -34) was similar. These data indicate recovery of the HC<sup>pro</sup>-transgenic plants from PVA infection (*sensu* Lindbo *et al.*, 1993).





**Fig. 2.** Analysis of virus distribution in the leaves at positions +6 and +7 displaying the LLT phenotype in HC<sup>pro</sup>-transgenic *N. benthamiana* plants inoculated with PVA isolate B11. (A) Plants were inoculated on the first fully expanded leaf (position 0). All leaves at positions +1 to +5 above the inoculated leaf were infected (the leaf to the left), showing severe symptoms of chlorosis (mosaic) and malformation at 14 days p.i. The leaves at positions +6 and +7 expressed a phenotype in which only the leaf tip was chlorotic (LLT phenotype). The leaves at position +8 and above were symptomless. (B, C) Quantification by ELISA of PVA CP (blue bar) and HC<sup>pro</sup> (orange bar) in the tip (a), central part (b1) and basal part (b2) of the leaves at positions +6 (B) and +7 (C), which displayed initial signs of recovery, i.e., the LTT phenotype. The values indicated are the mean ( $n=9$ ) from three independent experiments. Bars indicate SD. The virus detection limit in ELISA was ca. 6 ng of virions per gram of leaf tissue. The detection limit for HC<sup>pro</sup> was ca. 3 ng of the protein per gram of leaf tissue. (D) Northern blot analysis of RNA extracted from different parts of the LLT leaves [leaf parts shown in (a, b1 and b2) above] of two plants. Two samples from non PVA-infected HC<sup>pro</sup>-transgenic plants were included as controls (c1 and c2). A deoxyribose probe specific to the HC<sup>pro</sup>-encoding region was used. It detects both the viral genomic RNA (PVA) and transgene mRNA (tr). The amount of RNA (~15  $\mu$ g) loaded per lane was verified by ethidium bromide staining of ribosomal RNAs (bottom panel).



**Fig. 3.** Northern blot analysis of accumulation of the HC<sup>pro</sup> transgene mRNA (asterisk) in leaves at position -1 before PVA inoculation (sample 0; lanes 1, 3, 5, 7, 9 and 11) and by 28 days p.i. (sample 1; lanes 2, 4, 6, 8 and 12) in the recovered symptomless leaves at position +8 and in a mock-inoculated plant (lanes 13 and 14). Note that PVA genomic RNA was not detected 28 days p.i. due to degradation by the RNA silencing mechanism. The amount of RNA (~30  $\mu$ g) loaded per lane was verified by ethidium bromide staining of ribosomal RNAs (bottom panel).

Distribution of PVA and the steady-state levels of transgene mRNA expression were examined in different parts of the leaves displaying the LLT phenotype. The leaves from positions +6 and +7 on 10 recovered HC<sup>pro</sup>-transgenic plants and leaves from similar positions on a few healthy HC<sup>pro</sup>-transgenic plants (controls) were harvested and each leaf was divided into three parts (Fig. 2). The samples were analysed by ELISA and Northern blot (Fig. 2). Readily detectable amounts of the PVA CP antigen (Fig. 2B,C), transgene mRNA and PVA genomic RNA (Fig. 2D) were detected in the tips of the leaves showing the LLT phenotype. In contrast, the other parts of these leaves contained very low or undetectable amounts of the virus and transgene mRNA (Fig. 2B–D). The symptomless leaves at position +8 or higher did not contain detectable amounts of the virus or transgene mRNA (Fig. 3). In the non-infected HC<sup>pro</sup>-transgenic control plants, the leaves at positions +6 to +9 contained readily detectable amounts of the transgene mRNA, as tested at two time-points (Fig. 2D, lanes 1 and 14; Fig. 3, lanes 13 and 14).

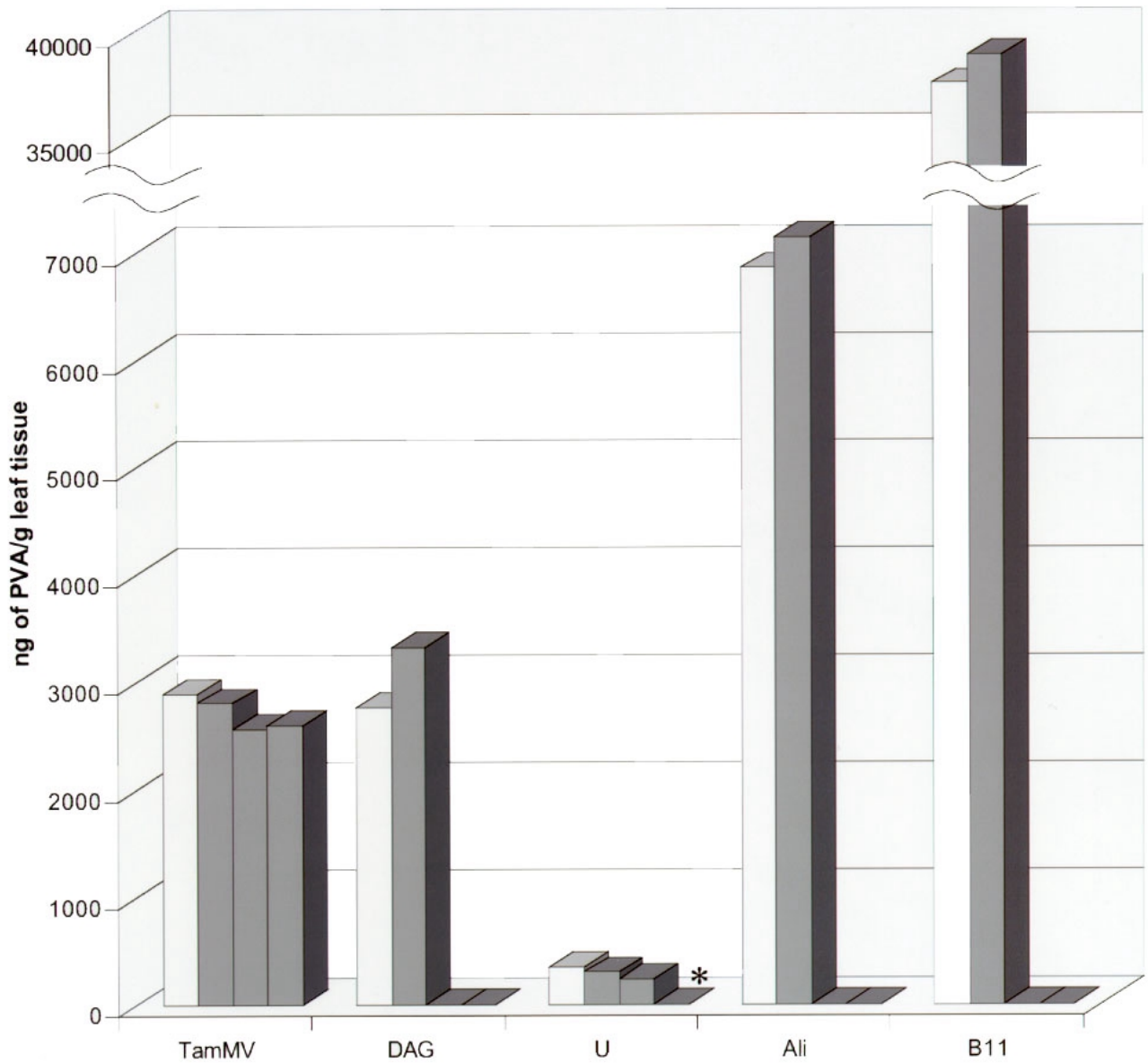
The recovered virus-free leaves of the HC<sup>pro</sup>-transgenic plants at position +8 or higher were challenged with PVA isolate B11 by mechanical inoculation. Six leaves of the 29 inoculated leaves (21 %) were infected in two experiments, as tested by ELISA at 14 days p.i. These data indicate that the recovered leaves were not completely resistant to PVA infection when mechanically inoculated. However, since the leaves at position +8 and above remained symptomless and virus-free in the recovered plants, these leaves were apparently resistant to the virus systemically transported from the infected leaves at positions 0–5. The controls in these experiments included CP-transgenic plants (10 leaves inoculated, no leaves infected) and GUS-transgenic plants (10 leaves tested, all leaves infected), showing responses consistent with previous studies (Savenkov & Valkonen, 2001b).

### **Responses to infection with different isolates of PVA**

Three additional PVA isolates (Ali, TamMV and U), differing in their HC<sup>pro</sup> and CP sequences compared to isolate B11 (Kekarainen *et al.*, 1999), were used for inoculation of the HC<sup>pro</sup>-transgenic plants. Also, a mutant of PVA isolate B11 (B11-DAG), bearing two nucleotide substitutions in the CP gene and accumulating to ca. 10-fold lower titres than B11 in tobacco plants (*N. tabacum* cv. Samsun) (Andrejeva *et al.*, 1999), was included in the experiments. Accumulation of these PVA isolates in *N. benthamiana* was first tested in wt and GUS-transgenic plants and found to be different. Isolate B11 (37.7 µg/g leaf, *n*=18) had very high titres. Isolate Ali (6.86 µg/g, *n*=13) had moderately high titres, TamMV (3.0 µg/g, *n*=15) and B11-DAG (2.76 µg/g, *n*=13) had low titres and isolate U (0.35 µg/g, *n*=21) had very low titres, as estimated by

ELISA on at least 13 inoculated plants per isolate. These differences in virus accumulation were consistent with the differences observed in tobacco plants (Rajamäki *et al.*, 1998; Andrejeva *et al.*, 1999; Valkonen *et al.*, 2002). The T1 progeny plants of the CP-transgenic line were shown previously to be resistant to all these PVA isolates, probably due to a PTGS-based resistance mechanism, as proposed previously (Table 1) (Savenkov & Valkonen, 2001b). In this study, these plants were compared to the HC<sup>pro</sup>-transgenic plants for their response to infection with the aforementioned PVA isolates and the mutant B11-DAG. No infection with any of these viruses was detectable in inoculated or upper non-inoculated leaves in any of nine plants per virus tested in two experiments (data not shown). Thus, the CP-transgenic plants expressed extreme resistance to a range of PVA isolates showing as low as 86.9 % sequence identity to the transgene (CP-encoding region; the 5' UTR sequences of TamMV and B11 are only 68.9 % identical) (Kekarainen *et al.*, 1999).

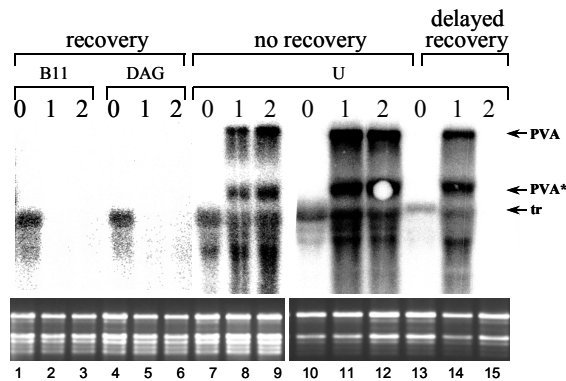
In contrast, initially, all HC<sup>pro</sup>-transgenic plants became systemically infected with the four PVA isolates and B11-DAG by 14 days p.i. and accumulated to titres not significantly different from the control plants (Fig. 4). Isolates B11, Ali and B11-DAG induced severe mosaic symptoms in the systemically infected leaves, whereas TamMV and U caused only mild mosaic symptoms. In plants infected with B11, B11-DAG and Ali, the leaves at positions +6 and +7 developed the LLT phenotype at 28 days p.i. and the leaves developing at higher positions were symptomless, showing recovery as before. In contrast, the leaves at positions +6 and above continued to show mild mosaic symptoms in plants infected with TamMV or U. However, by 38 days p.i., in nine plants out of the total of 19 plants infected with isolate U, the new leaves developed without symptoms and were virus-free (Fig. 4). These symptomless leaves in the nine plants and the corresponding symptomatic leaves of other plants infected with isolate U were compared for accumulation of transgene mRNA and PVA RNA (Fig. 5). A few leaves at position +9 from plants inoculated with isolate B11 or B11-DAG were included as well. No transgene mRNA or viral RNA was detected in the symptomless leaves, whereas transgene mRNA and viral RNA were detected in all symptomatic leaves (Fig. 5). Thus, isolate U induced a delayed recovery from infection, which was observed in only a few plants and was devoid of the LLT phenotype, in contrast to B11, B11-DAG and Ali. No recovery was observed in plants infected with TamMV. The data suggest that the PVA isolates showing higher sequence similarity with the transgene (B11, B11-DAG and Ali versus U and TamMV) and/or accumulating to higher titres (B11 and Ali versus U and TamMV) were more likely to induce recovery in the HC<sup>pro</sup>-transgenic plants.



Percentage nt. identity to the transgene	81.3%	100%	97.5%	99.4%	100%
Percentage recovered plants at 28dpi	0	100%	0	100%	100%
at 38dpi	0	100%	47%*	100%	100%

**Fig. 4.** The titre and sequence homology dependence of the recovery phenomenon (RNA silencing) in transgenic *N. benthamiana* plants overexpressing HC<sup>pro</sup> and infected with different PVA isolates. PVA accumulation was estimated by ELISA in the upper non-inoculated leaves including known amounts of purified PVA virions for comparison. The leaf at position +5 above the inoculated leaf in the non-transgenic control plants (open bars) and the HC<sup>pro</sup>-transgenic plants (closed bars) were tested by 14 days p.i. The HC<sup>pro</sup>-transgenic plants were also tested

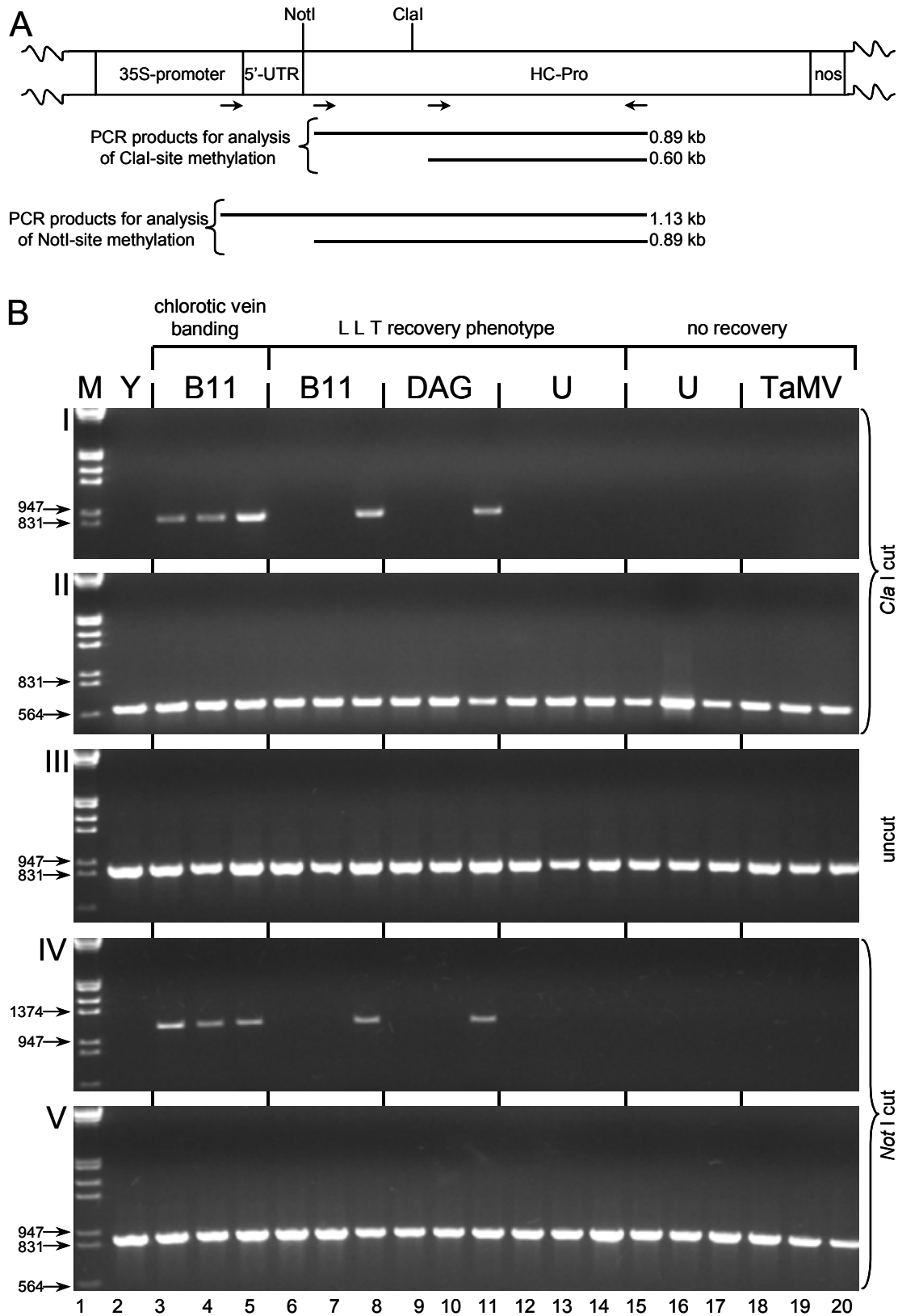
at two later time-points: 28 (leaf at position +9) and 38 (leaf at position +9) days p.i. PVA isolate TamMV with low (81.3 %) nucleotide sequence identity to the transgene did not induce recovery, in contrast to the three other isolates. Recovery induced by isolate U was delayed until 38 days p.i. and only 9 (\*) of 19 plants recovered. Data are combined from at least three independent experiments per virus isolate, each experiment included at least four plants per isolate.



**Fig. 5.** Northern blot analysis of accumulation of the HC<sup>pro</sup> transgene mRNA and PVA genomic RNA in transgenic plants undergoing recovery. Total RNA was extracted immediately before PVA inoculation (lanes 0) and by 28 (lanes 1) and 38 (lanes 2) days p.i. from the leaves at positions +8 and +9 above the inoculated leaf. PVA isolates B11, B11-DAG and U were used for inoculation. Genomic RNA of PVA is visible as a high molecular mass RNA (PVA) and several smaller bands (PVA\*). The transgene transcript is indicated (tr). An equal amount of RNA (~10 µg) was loaded per lane, as shown by ethidium bromide staining of ribosomal RNAs (bottom panel).

### Methylation of the HC<sup>pro</sup>-transgene DNA

Methylation of the HC<sup>pro</sup> transgene during recovery of plants from PVA infection was tested using a PCR-based technique employing methylation-sensitive endonucleases and subsequent PCR amplification (Ingelbrecht *et al.*, 1994; Guo *et al.*, 1999; Mallory *et al.*, 2001). Total DNA was isolated from young leaves before PVA inoculation and from the upper leaves at positions +7 and +8 above the inoculated leaf by 28 and 38 days p.i., respectively. DNA from young seedlings 5 days post-germination was used as a control for the methylation test. Two enzymes were used to provide two sets of independent reference digestions (Fig. 6A). To amplify three partially overlapping portions of the HC<sup>pro</sup> transgene, four primers were designed and used. The reverse primer complementary to the 3' proximal part of the transgene was used in combination with three forward primers (Fig. 6A). If the *Clal* (*NotI*) sites in an analysed transgene were methylated, then it would be expected that the enzymes would not be able to cut the genomic DNA and both a 0.89 kb (1.2 kb in the case of *NotI* site) and a 0.6 kb (0.89 kb) PCR product would be amplified. However, if the DNA was not methylated at those sites, then the enzymes would cut and only the small fragments of 0.6 kb (0.89 kb) would be amplified.



**Fig. 6.** Analysis of transgene methylation in PVA HC<sup>pro</sup>-transgenic plants upon infection with four PVA isolates. (A) Schematic representation of the HC<sup>pro</sup> transgene. The HC<sup>pro</sup> gene is flanked by the CaMV 35S promoter fused with the PVA 5' UTR and the nopaline synthase gene terminator sequence (nos). Restriction sites for *NotI* and *ClaI* are indicated. Sizes of the PCR products amplified following digestion with *NotI/ClaI* are shown and the positions of

PCR primers in relation of the transgene sequence are indicated with arrows. One reverse primer and three forward primers were used, of which the forward primer used for amplification of the 0.89 kb fragment was the same for digestions with both *NotI/ClaI*. (B) Agarose gel electrophoresis of the PCR products amplified from *ClaI*-digested (panels I and II), *NotI*-digested (panels IV and V) or non-digested (panel III) samples of total DNA. The digested DNA was amplified by two pairs of primers, in which the forward primer was located upstream (panels I and IV) or downstream (panels II and V) of the restriction site. Uncut DNA (panel III) was amplified using the forward primer, also used to amplify the 0.89 kb fragment. The reverse primer was the same for all PCR amplifications. The HC<sup>pro</sup>-transgenic plants were inoculated with four PVA isolates (B11, B11-DAG, U and TamMV). Upper leaves were analysed prior to PVA inoculation (lanes 3, 6, 9, 12, 15 and 18) and the leaves at positions +9 and +10 above the inoculated leaf were analysed by 28 (lanes 4, 7, 10, 13, 16 and 19) and 38 (lanes 5, 8, 11, 14, 17 and 20) days p.i. The recovery phenotypes are indicated on the top (see text for detailed description). A DNA sample from young seedlings (5 days post-germination) of the HC<sup>pro</sup>-transgenic plants was included as a control (lane 2). Lane 1 in each panel shows the  $\lambda$  *EcoRI/HindIII* molecular mass marker, of which the sizes of two marker bands are indicated.

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Results of the analysis for methylation within the transgene DNA indicated that of the *ClaI* (*NotI*)-digested DNA samples from young seedlings, from plants prior to PVA inoculation and from the recovered leaves at 28 days p.i. produced the pattern predicted for unmethylated DNA, since the large fragments were not amplified (Fig. 6B, panels I and IV), whereas the small fragments (Fig. 6B, panels II and V) as well as the expected fragment from undigested DNA (Fig. 6B, panel III) were amplified.

At 38 days p.i., the pattern predicted for methylated DNA was observed for recovered leaves of plants inoculated with PVA isolates B11 or B11-DAG but not in the recovered leaves of plants inoculated with isolate U (Fig. 6B, panels I and IV) (plants inoculated with isolate U showed recovery by 38 days p.i., 10 days later than the plants inoculated with B11 or B11-DAG). Methylation of the HC<sup>pro</sup> transgene was not detected in the plants inoculated with isolates U or TamMV and which did not undergo recovery but showed disease symptoms in the upper leaves by 38 days p.i.

A single plant among the over one hundred T1 plants of line ab34 tested in our experiments showed a unique phenotype. Recovery of the upper leaves started 1 week earlier than in other plants and chlorotic vein banding in lower leaves as well as the LTT phenotype developed in this plant. In addition, the steady-state levels of HC<sup>pro</sup> mRNA (Fig. 3, lane 11) were somewhat lower than in other plants prior to PVA inoculation. We found that *ClaI* (*NotI*)-digested DNA produced the pattern of amplification predicted for methylated DNA at all three time-points: amplification of both the long (Fig. 6B; panels I and IV, lanes 3–5) and the short (Fig. 6B; panels II and V, lanes 3–5) fragments was observed.

Taken together, these results suggest that these particular *ClaI* and *NotI* sites located 300

nt from each other were not methylated during the initiation and the early maintenance stage of silencing (recovery) but became methylated to some extent at the later maintenance stage. Isolates unable to induce recovery and silencing of the transgene failed to induce methylation of the homologous sequence within a transgene. On the other hand, early methylation of the coding sequence of the transgene was associated with a distinct rare recovery phenotype ('chlorotic vein banding' sample; Fig. 6B, lanes 3–5).

## Discussion

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RNA silencing constitutes a natural defence mechanism against viruses in plants (Covey *et al.*, 1997; Ratcliff *et al.*, 1997; Al-Kaff *et al.*, 1998). Thus, it is conceivable that transgenic plants expressing an RNA silencing suppressor, such as the potyvirus HC<sup>pro</sup>, would be compromised for virus resistance and converted to hypersusceptibility to virus infection. Indeed, expression of the P1/HC<sup>pro</sup> polyprotein of *Tobacco etch virus* (TEV) in transgenic tobacco plants enhances accumulation of unrelated viruses in these plants (Vance *et al.*, 1995). Also, the titre of *Potato leafroll virus* is significantly increased in the PVA HC<sup>pro</sup>-transgenic *N. benthamiana* plants (Savenkov & Valkonen, 2001a). However, it was of particular interest to investigate how transgenic plants overexpressing an RNA silencing suppressor would respond to infection with viruses homologous to the transgene. In such plants, the anti-silencing force conferred together by the transgene product and the silencing suppressor produced from the replicating virus would be encountered by a silencing induction force created by dsRNA, a potent inducer of RNA silencing (Chicas & Macino, 2001) produced during virus replication (virus-induced gene silencing, VIGS) (Baulcombe, 1999). Our study shows that the force of VIGS overcomes the silencing suppression potential conferred by the combined production of HC<sup>pro</sup> from the transgene and replicating PVA in the plants used in this study. Interestingly, only brief, transient recovery was observed in another recent study in which *N. benthamiana* plants transformed with the HC<sup>pro</sup> gene of *Cowpea aphid-borne mosaic virus* were challenged with the homologous virus (Mlotshwa, 2001).

The HC<sup>pro</sup>-transgenic *N. benthamiana* plants used in this study express high levels of HC<sup>pro</sup> (300–900 ng HC<sup>pro</sup> per gram of leaf, as compared to 500–1700 ng/g accumulating in wt plants infected with PVA isolate B11) (Savenkov & Valkonen, 2001a). No spontaneous silencing of the HC<sup>pro</sup> transgene has been observed in any of the several hundred T1 progeny plants examined from several transgenic lines. Also, all examined plants were initially susceptible to PVA infection. The HC<sup>pro</sup>-transgenic plants of this study showed no morphological abnormalities, in contrast to another study on TEV HC<sup>pro</sup> transgenic plants (Anandalakshmi *et*

*al.*, 2000), possibly due to differences in the viruses or transgene constructs used.

Replication of PVA following systemic spread to newly developing tissues results in a rapid increase of the PVA RNA concentration in the cells. However, meristematic tissues that lack functional plasmodesmal connections and very young leaf primordia which lack veins and phloem tissues supportive to virus transport and unloading (Roberts *et al.*, 1997) cannot be systemically infected following phloem-dependent virus transport from other parts of the plant (Oparka & Santa Cruz, 2000). The initiation of recovery (LLT phenotype) was consistently observed in two successive new leaves, in which the tips were found to be virus-infected but the rest of the leaf was symptomless and virus-free. The tip is developmentally the oldest part of the leaf (Roberts *et al.*, 1997). Thus, it seems likely that RNA silencing is initiated in close proximity to the meristematic tissues. Indeed, meristems do not undergo RNA silencing (Beclin *et al.*, 1998; Voinnet *et al.*, 1998) and potyviruses do not access the meristem (Jones *et al.*, 1998b).

A spatial pattern of RNA silencing resembling the LLT phenotype observed in this study is induced by infection with a homologous virus in transgenic pea plants expressing NIB, the potyviral RNA-dependent RNA polymerase derived from PSbMV (Jones *et al.*, 1998b). Recovery from PSbMV infection was first observed in the pea leaves at positions +3 and +4 above the inoculated leaf, similar to leaves at positions +6 and +7 in our study, and was characterized by a restriction of symptoms and virus accumulation to the distal part of the leaf. Furthermore, the leaf at position +5 and the subsequent new leaves, similar to the leaf +8 and the subsequent new leaves in our study, were resistant to mechanical inoculation with PSbMV (Jones *et al.*, 1998b). In the PSbMV NIB-transgenic pea plants and the PVA HC<sup>pro</sup>-transgenic *N. benthamiana* plants, the rest of the leaf lamina showed recovery. However, the leaves of *N. benthamiana* plants transformed with the NIB gene of *Plum pox virus* (Guo & Garcia, 1997) show an irregular pattern of dark green islands ('bubbles') within otherwise chlorotic leaf lamina, indicating a sporadic induction of RNA silencing in the leaf lamina (Moore *et al.*, 2001).

Once recovered, the new top leaves did not become systemically infected with PVA transported from the lower, full-grown, PVA-infected source leaves. However, six leaves of the 29 recovered leaves mechanically inoculated with the homologous PVA isolate B11 were infected, showing that the recovered leaves were not completely protected against PVA infection. Apparently, mechanical inoculation of PVA can circumvent the mechanism that prevented infection via systemic, phloem-dependent virus transport from the lower leaves. These data are consistent with the hypothesis that RNA silencing may be hyperactive in phloem cells that control phloem-loading and/or phloem-unloading of viruses (Marathe *et al.*, 2000). Thus, effective degradation of viral RNA upon infection of the phloem cells could inhibit

systemic infection, whereas in the mechanically inoculated leaves, the gate-keeper function of the phloem cells would be circumvented.

A threshold model of RNA silencing predicts that the concentration of silencing target RNA is reduced to just below a threshold level in leaves where silencing has been initiated (Meins, 2000). It is intriguing in this regard that in the lamina of +6 leaves, PVA accumulated at greatly reduced but detectable titres, which are, in quantitative terms, similar to the titres of PVA-U, an isolate that was able to induce only delayed recovery in some of the infected plants. These findings suggest that viruses replicating to only low titres and/or accumulating low amounts of replicative intermediates (dsRNA) do not reach a threshold level and can evade and/or do not induce the plant RNA surveillance system. However, because no virus and no transgene mRNA were detected in the leaves at position +8 or higher, our data suggest that another mechanism independent of the threshold level contributes to the propagation and maintenance phases of silencing.

Our data indicate that the level of sequence homology between the transgene and the infecting virus constitutes an important factor in induction of recovery. Isolates B11 and B11-DAG are identical to the transgene sequence and induced recovery to the same rate regardless of a more than 10-fold difference in the level of virus accumulation. However, isolate TamMV with a low nucleotide sequence identity to the transgene (81.3 % or, more specifically, 68.9 % for the 5' UTR and 83.2 % for the HC<sup>pro</sup> cistron) (Kekarainen *et al.*, 1999) and an accumulation level similar to isolate B11-DAG induced no recovery (Fig. 4). Isolate U accumulated to titres ca. 10-fold lower than B11-DAG and TamMV, but it induced a delayed recovery in 47.4 % of infected plants, probably due to its high levels of sequence similarity with the transgene (97.5 %) (Fig. 4). In contrast to the HC<sup>pro</sup>-transgenic plants, the CP-transgenic plants were resistant to all five PVA isolates tested and no infection was observed. The sequence identity between TamMV and the CP transgene is 86.9 %, which is slightly higher than the identity between TamMV and the HC<sup>pro</sup> transgene. Taken together, our data suggest that higher levels of virus accumulation and sequence similarity with the transgene both represent factors of great importance for RNA silencing induction by a virus (VIGS).

Methylation of the transgene is often found to be associated with the maintenance phase of RNA silencing (Jones *et al.*, 1998b, 1999; Guo *et al.*, 1999). In order to address the possible role of transgene methylation on RNA silencing in the HC<sup>pro</sup>-transgenic plants, methylation was assayed at two restriction sites located ca. 300 nt apart at different times after infection with five PVA isolates. No indication of methylation was apparent at the time when many leaves of the plants had recovered from PVA infection. Even though methylation of the transgene was later

observed in recovered leaves, its appearance at a late stage of recovery indicated no significant role in the initiation phase of recovery but rather a possible significance in the maintenance stage of transgene silencing (Jones *et al.*, 1999).

The results of our study are consistent with the previous observations that many viruses encoding functional suppressors of RNA silencing will, nevertheless, trigger RNA silencing during infection (Carrington *et al.*, 2001) and are, therefore, both suppressors and targets of the silencing (Voinnet *et al.*, 1999).

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