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# Expression of self-complementary hairpin RNA under the control of the *rolC* promoter confers systemic disease resistance to plum pox virus without preventing local infection

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## Abstract

**Background:** Homology-dependent selective degradation of RNA, or post-transcriptional gene silencing (PTGS), is involved in several biological phenomena, including adaptative defense mechanisms against plant viruses. Small interfering RNAs mediate the selective degradation of target RNA by guiding a multicomponent RNAse. Expression of self-complementary hairpin RNAs within two complementary regions separated by an intron elicits PTGS with high efficiency. Plum pox virus (PPV) is the etiological agent of sharka disease in *Drupaceae*, although it can also be transmitted to herbaceous species (e.g. *Nicotiana benthamiana*). Once inside the plant, PPV is transmitted via plasmodesmata from cell to cell, and at longer distances, via phloem. The *rolC* promoter drives expression in phloem cells. *RolC* expression is absent in both epidermal and mesophyll cells. The aim of the present study was to confer systemic disease resistance without preventing local viral infection.

**Results:** In the *ihprolC-PP197* gene (*i*ntron *h*air *p*in *rolC PPV 197*), a 197 bp sequence homologous to the PPV RNA genome (from base 134 to 330) was placed as two inverted repeats separated by the DNA sequence of the *rolA* intron. This hairpin construct is under the control of the *rolC* promoter.*N. benthamiana* plants transgenic for the *ihprolC-PP197* gene contain siRNAs homologous to the 197 bp sequence. The transgenic progeny of *ihprolC-PP197* plants are resistant to PPV systemic infection. Local infection is unaffected. Most (80%) transgenic plants are virus free and symptomless. Some plants (20%) contain virus in uninoculated apical leaves; however they show only mild symptoms of leaf mottling. PPV systemic resistance cosegregates with the *ihprolC-PP197* transgene and was observed in progeny plants of all independent transgenic lines analyzed. SiRNAs of 23–25 nt homologous to the PPV sequence used in the *ihprolC-PP197* construct were detected in transgenic plants before and after inoculation. Transitivity of siRNAs was observed in transgenic plants 6 weeks after viral inoculation.

**Conclusions:** The *ihprolC-PP197* transgene confers systemic resistance to PPV disease in *N. benthamiana*. Local infection is unaffected. This transgene and/or similar constructs could be used to confer PPV resistance to fruit trees where systemic disease causes economic damage.

## Background

Plants use post-transcriptional gene silencing (PTGS) as a mechanism of adaptive protection against viruses [1]. PTGS is closely related to RNA interference and, consequently, represents an ancient eukaryotic genetic phenomenon [2]. PTGS correlates with the presence of small interfering RNAs (siRNAs) [3]. These latter molecules are composed of 21–26 ribonucleotides that originate from double-stranded RNA (dsRNA) by the action of Dicer RNAses [4][5]. dsRNA can be generated by several mechanisms that include host or virus-encoded RNA-dependent RNA polymerases, transcripts bearing inverted repeats, and transcription of complementary DNA strands. siRNAs are a component of the RNA-induced silencing complex (RISC) [6]. RISC ribonuclease activity degrades RNA homologous to siRNAs [7]. siRNAs homologous to the target RNA guide selective RNA degradation[7]. Thus, siRNAs confer specificity to the ribonuclease activity of RISC [3][6][7].

Post-transcriptional gene silencing acts intracellularly. However, the RNA silencing state can be transmitted from a plant cell to its neighbour via plasmodesmata and at longer distances via the vascular system [8]. In plants, it has been proposed that the 21–22 nt-long siRNAs are involved in the selective intracellular degradation of RNA, while the longer (24–26 nt) siRNAs are involved in systemic signaling of PTGS and/or other aspects of RNA silencing [9][10][11].

PTGS has been achieved with high efficiency in transgenic plants expressing self-complementary hairpin RNA (hpRNA) [12][13]. hpRNAs have two complementary regions that form a double-stranded region separated by a short loop. Hairpin constructs having a spliceosomal intron inserted in the loop region (ihpRNA) show an efficiency of up to 90% in eliciting RNA silencing [13].

Plum pox *potyvirus* (PPV) is the etiological agent of sharka, a severe plant disease that infects *Drupaceae*, such as peach, apricot, plum, cherry and almond [14]. Systemic infection by PPV impairs plant growth and drastically curtails fruit productivity and quality [14][15]. PPV may be transmitted either by aphids in a non-persistent manner or by grafting [15]. The PPV genome consists of a positive single-stranded RNA of approximately 10 kb [16]. Viral replication begins in infected epidermal cells and then, via plasmodesmata, the virus reaches mesophyll cells, bundle sheaths, phloem parenchyma and companion cells [17]. Long distance movement occurs through the phloem sieve-tubes and causes rapid systemic spreading of the infection within the plant [17]. Resistance to PPV disease has been achieved by PTGS in one of 5 independent peach plants transgenic for the PPV coat protein expressed under the control of the constitutive 35S CaMV promoter [18]. Other approaches have been used to obtain transgenic plants transformed with PPV sequences in herbaceous species [15]. Fully resistant *N. benthamiana* plants have been obtained in one of 7 lines transgenic for a mutated CI protein [19]. *N. benthamiana* plants transgenic for mutated DNA sequences corresponding to the NIa-NIb and N-terminus of the capsid protein, showed virus resistance and, depending on the specific mutations, in all or in a fraction of the plants tested [20].

We hypothesised that if systemic movement of PPV could be impaired, the virus should remain confined to inoculated leaves and, consequently, would not cause systemic disease. In this regard, primary inoculated leaves never show the severe symptoms that characterise systemic infection by potyvirus. Furthermore, the number of primary infection foci determined by aphid transmission does not extensively affect plant growth and productivity. Moreover, PTGS is an adaptative mechanism of the defence where the virus genome is both target for degradation and substrate for amplification of the PTGS response. Thus, in our opinion, the enduring presence of viruses in locally infected leaves might boost PTGS.

Thus, we have tested whether systemic resistance to PPV disease can be conferred by an hpRNA gene construct that would be predicted to be processed by Dicer RNAse(s) to siRNAs. siRNAs homologous to the PPV genome would then trigger the degradation of PPV RNA. To drive expression of the hpRNA construct, we have used the *rolC* promoter from *Agrobacterium rhizogenes* [21][22]. The *rolC* gene, and consequently the *rolC* promoter, is not expressed in either epidermal or mesophyll cells, but is expressed in cells of the vascular system. In *Nicotiana tabacum*, by using a *rolC-uidA* reporter gene, *rolC* driven expression was localised to cells of the phloem vascular system [21][22]. In the same species, using anti-rolC affinity purified antibodies, *rolC* expression was localised in companion and protophloem cells [23]. In potato, the *rolC* promoter has been used to confer viral resistance to potato leafroll virus (PLRV) by expressing the PLRV coat protein under its control [24]. In this species, by using a *rolC-uidA* reporter gene in a strongly expressing transgenic plant, *rolC* driven expression was also observed in bundle sheath and vascular parenchyma cells, but not in epidermal or mesophyll cells [24].

HairpinRNA gene constructs in which the 2 complementary regions of the hpRNA separated by an intron are extremely efficient in triggering PTGS [13]. In the *ihprolC-PP197* (intron-hairpin *rolC* PPV 197) gene construct used for the present experiments, the DNA sequence corresponding to the *rolA* intron was inserted between the two sequences derived from the PPV genome that build the double-stranded region of the hpRNA. The *rolA* intron of

*A. rhizogenes* is 85 bases long and is spliced in both *Arabidopsis*[25] and *Nicotiana tabacum* [26]. *Agrobacterium rhizogenes* causes hairy-root disease in many plant species [27]. Consequently, the *rol* genes are likely to be widely expressed among plants. Thus, the *rolC* promoter and the *rolA* intron should provide the genetic information necessary for the construction of a hpRNA that would be expressed in most, if not all, plant species. Here, we show that the *ihprolC-PP197* gene confers systemic PPV resistance to *Nicotiana benthamiana* plants. Consistent with the known expression pattern of the *rolC* promoter, PPV infection and replication are not prevented in inoculated leaves of *ihprolC-PP197* transgenic plants. Local symptoms develop in inoculated leaves, although systemic PPV disease is either prevented or drastically curtailed.

## Results

### *Rationale and design of the* ihprolC-PP197 *transgene*

Expression of the *ihprolC-PP197* transgene (Fig. 1, panel A) was placed under the control of the *rolC* promoter from *Agrobacterium rhizogenes*. The *rolC* promoter is located in the intergenic region between the *rolC* and the *rolB* T-DNA genes of *A. rhizogenes* and spans 850 bp upstream of the transcriptional initiation site of *rolC* [28]. In the *ihprolC-PP197* gene, an 1182 bp DNA fragment spanning the entire intergenic region was used to drive gene expression. However, shorter DNA fragments might be used as well (e.g. the 850 bp long *Hind*III/*Hpa*I DNA fragment [24][28]). The *rolC* promoter drives vascular-specific expression. *RolC* gene expression takes place mainly in companion cells [23] and is also present in other phloem cell types [24], but is absent in epidermal and mesophyll cells [23][24]. The transcribed region of the *ihprolC-PP197* transgene is composed of 2 DNA sequences placed in inverted orientation in order to produce a self-complementary dsRNA molecule. Each of the 2 sequences consists of 197 bases derived from the genome of PPV strain D. The 197 bp DNA sequence corresponds to bases 134 to 330 of the PPV genome (accession number X16415). Thus, its sequence begins 13 bases before the AUG initiation codon of the PPV polyprotein at nucleotide 147 and ends 181 bases after the translational start site. The 197 base-long region shows 99.5% homology to the corresponding sequence of PPV strain M (the so-called PPV epidemic strain). An 87 bp-long DNA sequence containing the *rolA* intron of *A. rhizogenes* was placed between the two 197 bp complementary sequences (Fig. 1, panel A). Transcriptional termination and polyadenylation signals are present in a 251 bp DNA sequence derived from the *rolC* gene. The DNA sequence of the *ihprolC-PP197* is shown in Figure 1, panel B. Thus, in transgenic plants, after splicing the pre-mRNA transcribed from the *ihprolC-PP197* gene produces a mRNA with 2 virus-derived self-complementary regions of 197 bases each, separated by 14 bases that are predicted to form a loop of 10 bases.

### *Transgenic state of* ihprolC-PP197 Nicotiana benthamiana *plants*

The transgenic state of independent *Nicotiana benthamiana* transformation events was analyzed by Southern blotting. The 5 independent transgenic plants (T0 primary transformants) have either one (lines 13 and 29; Fig. 2, panel A, lanes 4 and 2, respectively), two (line 8; Fig. 2, panel B, lane 1), or several copies (i.e. at least 5 in line 9 and at least 4 in line 2; Fig. 2, panel A, lanes 3 and 5, respectively) of the *ihprolC-PP197* transgene per haploid genome. It is worthwhile mentioning that plant line 13 has a *ihprolC-PP197* transgene deleted of the region upstream of the *Hind*III site positioned 850 bp upstream the *rolC* initiation of transcription (Fig. 2, panel A, lane 4). Consequently, the deletion does not involve *rolC* promoter sequences.

The T0 plants were backcrossed with wild-type plants and the T1 progeny were analyzed for kanamycin resistance (data not shown). The transgenic state of the T1 progeny were either confirmed by PCR (data not shown) or characterised by Southern blot analysis (Fig. 2, panel B). In summary, on the basis of Southern blot analysis (Fig. 2 and data not shown) for either the *ihprolC-PP197* transgene or the *nptII* (kanamycin resistance) gene, in addition to segregation analysis for kanamycin resistance, the following conclusions can be drawn. Plant line 13 had one copy of the *nptII* gene and one copy of the *ihprolC-PP197* transgene. Plant line 29 had one copy of the transgene and 2 copies of the *nptII* gene, with one of the 2 *nptII* genes unlinked to the *ihprolC-PP197* transgene. In plant line 8, kanamycin resistance segregated as 2 loci. Southern blot analysis of the kanamycin resistant progeny of line 8 confirmed the segregation data of 2 unlinked T-DNA insertions, each one containing one *ihprolC-PP197* and one *nptII* gene (Fig. 2, panel B, lanes 2–12). In the progeny of plant line 9, kanamycin resistance segregated as at least 4 independent loci, while Southern blot analysis of the T0 primary transformant for either *nptII* (data not shown) or *ihprolC-PP197* (Fig. 2, panel A, lane 3) genes indicated the presence of at least 5 copies per haploid genome. In plant line 2, kanamycin resistance segregated as 5 loci, while Southern blot analysis (of the T0 primary transformants) showed the presence of at least 4 copies of the *ihprolC-PP197* gene (Fig. 2, panel A, lane 5).

### *RNA analysis of* ihprolC-PP197 *transgenic plants*

The 5 independent T0 transformants were analyzed for the presence of *ihprolC-PP197* mRNA (Fig. 3, panel A). In each plant, Northern blot analysis showed a mRNA of the expected size, i.e. approximately 700 bases (Fig. 3, panel A, lanes 2–6). Thus, all 5 independent transgenic plants (T0) expressed the *ihprolC-PP197* transgene, albeit at rather low steady-state levels for *rolC* driven expression. In fact, the steady-state level of *ihprolC-PP197* mRNA was 5

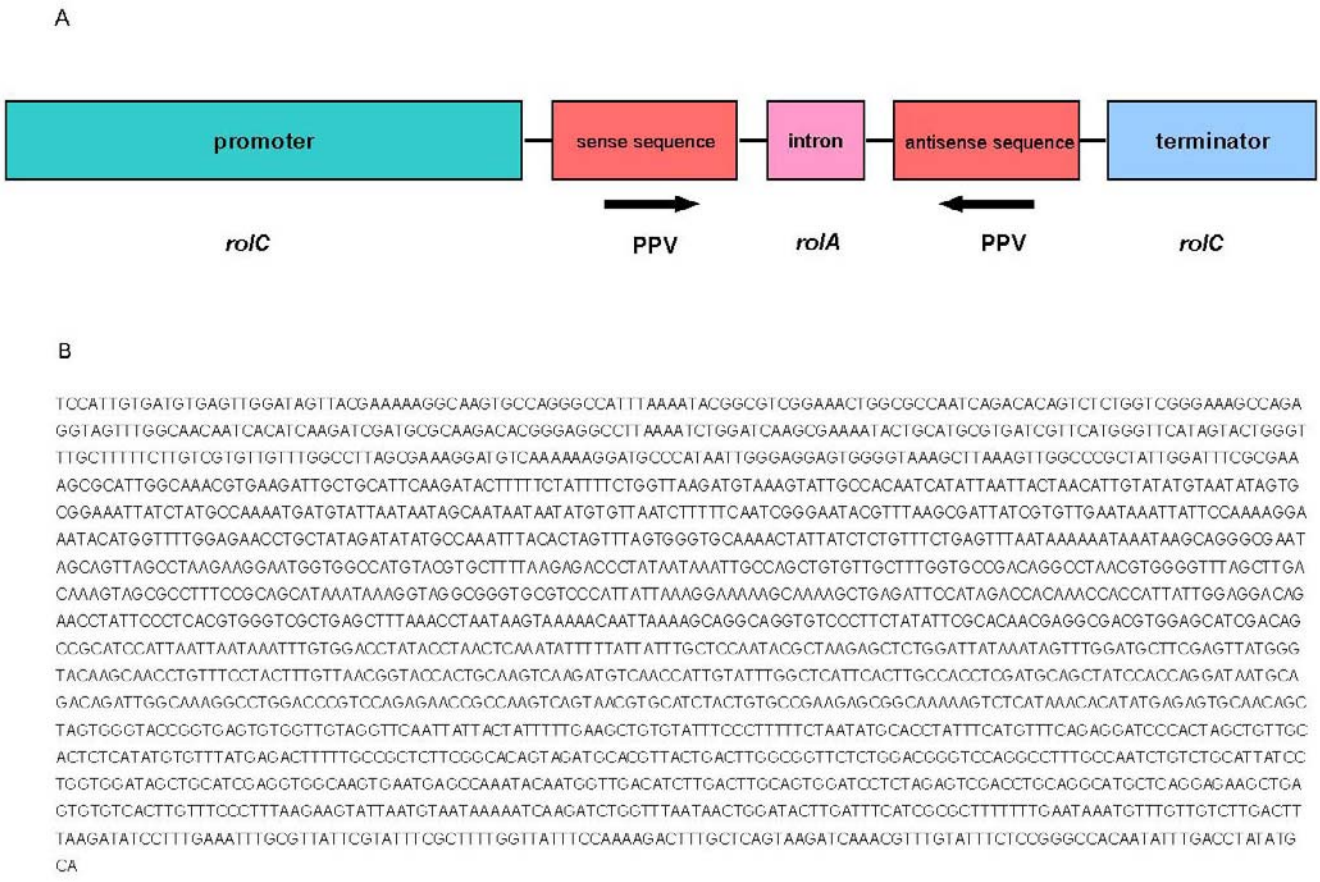


**Figure 1**
Panel A. Schematic drawing of *ihprolC-PP197* gene construct. Panel B. Nucleotide sequence of *ihprolC-PP197* gene construct.

to 10 times lower than that of a transgene (*rolC-PP2x197*) composed of the same regulatory and transcribed regions of the *ihprolC-PP197* construct, but bearing the 197 bp viral sequences repeated in a direct orientation (Fig. 3, panel B, lanes 2–4). These 5 independent T0 transgenic plants were analyzed for the presence of siRNAs. All 5 plants contained siRNAs homologous to the 197 base sequence derived from PPV (Fig. 4, panel A, lanes 2–6).

The transgenic T1 plants (backcrossed with wild-type plants) were also analyzed by RNA blotting for the presence of siRNAs. Northern blot analysis showed the presence of siRNAs homologous to the 197 bp sequence derived from the PPV genome (e.g. plant lines 2, 9, 13; Fig. 4, panel B, lanes 2, 3 and 4, respectively). Two siRNAs classes of 23 and 25 nt were detected with riboprobes derived from both strands of the 197 base long fragment of the PPV genome used to build the hairpin construct (Fig. 4, panel B and data not shown). Thus, *ihprolC-PP197* transgenic plants (T1) contain siRNAs homologous to the 197 base sequence derived from the PPV genome present in the hpRNA.

### Nicotiana benthamiana *plants transgenic for the* ihprolC-PP197 *gene are resistant to systemic PPV disease*

PPV can be experimentally transmitted to a range of host plants by mechanical inoculation [29]. The transgenic progeny (T1) of *ihprolC-PP197 Nicotiana benthamiana* plants were mechanically inoculated with PPV (D-serotype) on one-half of 3 basal leaves for each plant. Susceptibility or resistance to PPV infection was evaluated by monitoring symptoms of both local and systemic infection. For each of the 5 transgenic progeny, 5 to 11 individual transgenic plants were analyzed. Within 6–7 days after inoculation, inoculated half leaves of both transgenic and control plants developed chlorotic lesions (not shown). T1 backcrossed progeny were chosen in order to be able to evaluate virus resistance at the hemizigous state of single copy transgenes.

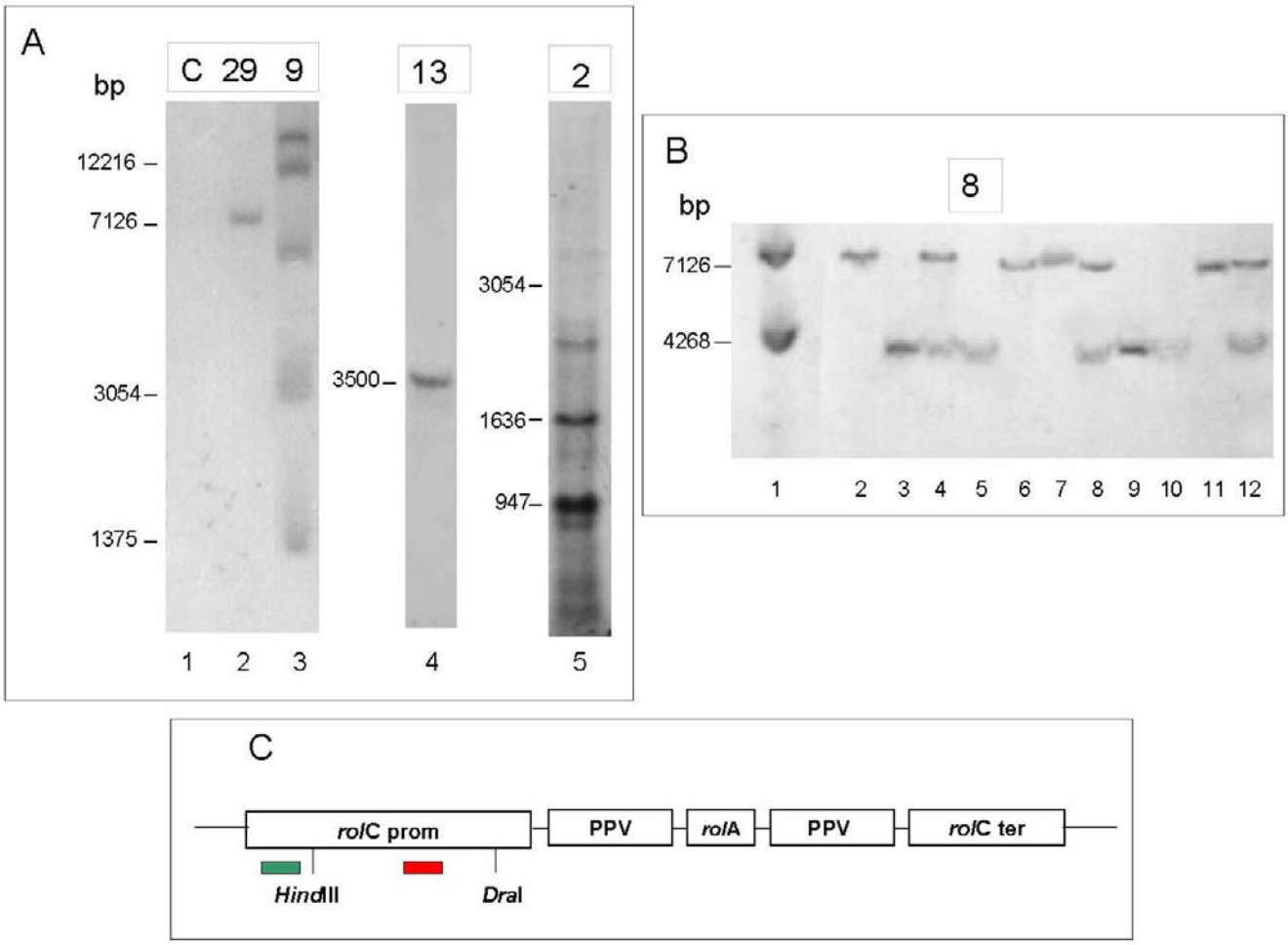


**Figure 2**
Southern blot analysis of *ihprolC-PP197 N. benthamiana* plants. Panel A. Transgenic state of T0 independent lines 2, 9, 29 and 13. Genomic DNA from control untransformed plant (lane 1) and transgenic lines 29, 9 and 2 (lanes 2, 3 and 5, respectively) was digested with *Hind*III; genomic DNA from transgenic line 13 (lane 4) was digested with *Dra*I. Panel B. Transgenic state of line 8. Genomic DNA from T0 line 8 (lane 1) and T1 progeny kanamycin resistant plants (lanes 2–12) was digested with *Hind*III. Panel C. Schematic drawing of the construct showing the position of the probes used in the Southern blot analysis. The probes used for lines 2, 9, 29, 8 and for line 13 are indicated with green and red boxes, respectively. Only restriction sites relevant for Southern analysis are shown. Expected bands for lines 2, 9, 29 and 8 should be longer than 620 bp. In line 13, the expected band should be higher than 1570 bp, however the integration event has removed approximately 600 bp from the T-DNA LB.

Wild-type control plants already showed severe systemic symptoms of disease at 6–7 days following inoculation, consisting in vein clearing and curling of the apical leaves (Fig. 5, panel A) and wilting (Fig. 5, panels B and C). None of the transgenic segregants from any of the 5 independent lines tested showed any symptom of disease (Fig. 5, panel C). Both plant growth and size were identical to control plants, either healthy wild-type or mock-inoculated transgenic plants (Fig. 5, panel D).

Wild-type plants were either dead or severely sick at 15 days after inoculation (Fig. 6, panel A). In contrast, the *ihprolC-PP197* transgenic progeny of plant line 13, 2 and 9 did not show any symptoms of systemic infection during their entire life cycle. All the plants grew normally, developed flowers (Fig. 6, panel B), and later set fruits with normal seeds (Fig. 6, panel C). Most (13 of 19) transgenic plants of the progeny of plant lines 8 and 29 did not show any symptoms of systemic disease during their entire life cycle. However, 20–25 days after PPV

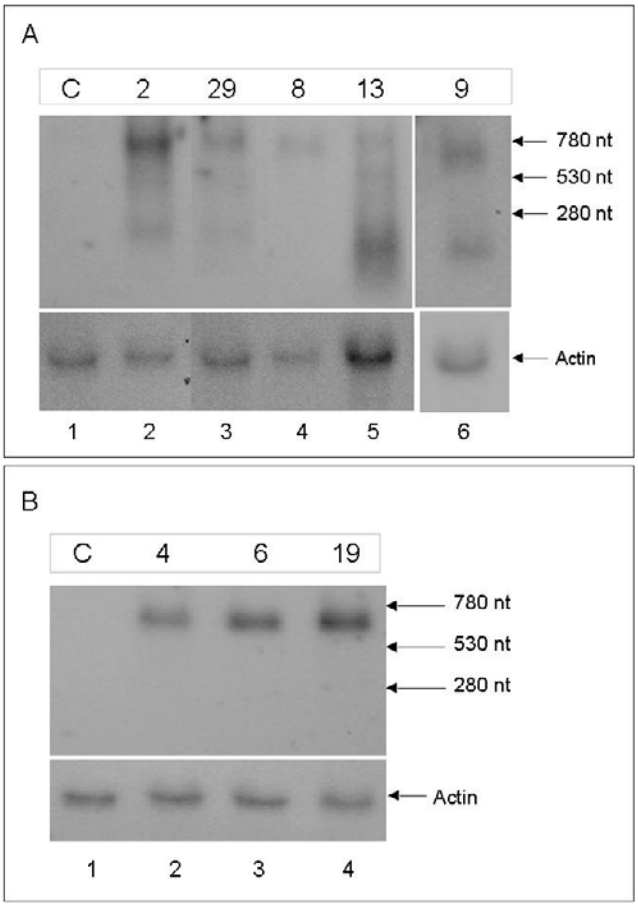


**Figure 3**
Northern blot analysis of *ihprolC-PP197* and *rolC-PP2x197* transgenic plants. Panel A. RNA blot of the *ihprolC-PP197* m*RNA* in *N. benthamiana* transgenic lines 2, 29, 8, 13 and 9 (lanes 2, 3, 4, 5 and 6, respectively) and control untransformed plant (lane 1). Panel B. RNA blot of *rolC-PP2x197* mRNA in *N. benthamiana* transgenic lines 4, 6 and 19 (lanes 2, 3 and 4, respectively) and control untransformed plant (lane 1). The blots were hybridised to a DNA probe corresponding to a 484 bp long sequence at the 3' end of the *ihprolC-PP197* gene. The *ihprolC-PP197* and *rolC-PP2x197* transcripts were detected after 3 days and 12 h of autoradiography, respectively. The membranes were also hybridised to an actin probe and exposed for 12 h. Both probes were labeled with ($\alpha$-$^{32}$P)-dCTP (3000 Ci/mmol) using "Ready to go DNA labeling beads (-dCTP)" (Amersham Biosciences); $10^6$ cpm/ml of labeled probe were added to the hybridization buffer.

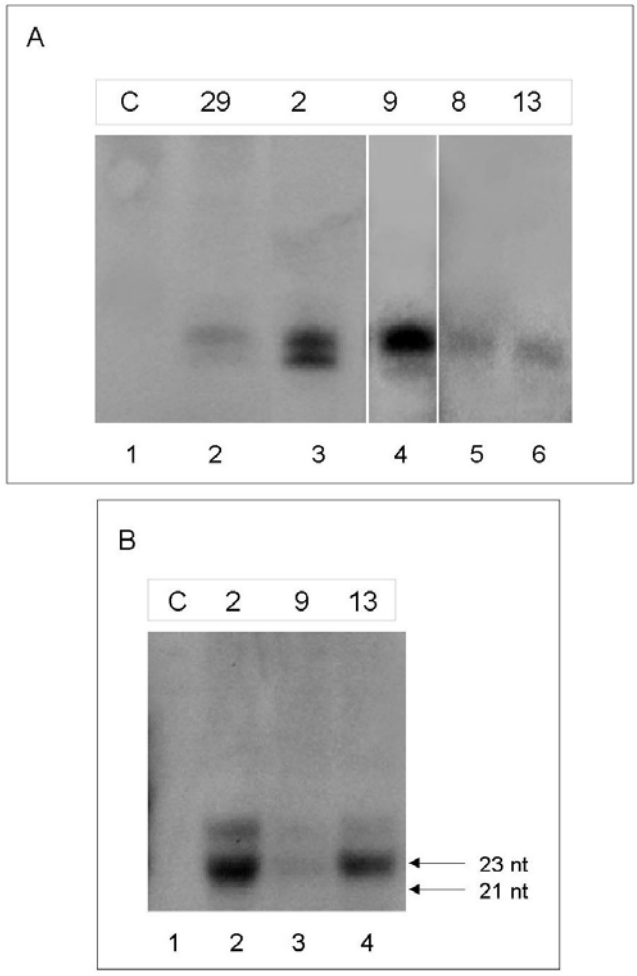


**Figure 4**
RNA gel blot analysis of siRNAs in *ihprolC-PP197* transgenic plants. Panel A. Northern blot analysis of siRNAs in T0 plants. Low molecular weight RNA was isolated from control untransformed plant (lane 1) and transgenic lines 29, 2, 9, 8 and 13 (lanes 2, 3, 4, 5 and 6, respectively). Panel B. Northern blot analysis of siRNAs in T1 plants. Low molecular weight RNA isolated from untransformed control plant (lane 1) and transgenic lines 2, 9 and 13 (lanes 2, 3 and 4, respectively). Approximatly 40 $\mu$g of low molecular weight RNA per sample was loaded. SiRNAs were detected by hybridisation with hydrolised single stranded $^{32}$P-labeled RNA probe corresponding to the 134–330 region of the PPV genome. 21 and 23 nt-long RNA oligoribonucleotides were used as molecular standards.

inoculation, some plants (3 of 11 transgenic plants from the progeny of line 8 and 3 of 8 transgenic plants from the progeny of line 29) showed mild symptoms of virus infection consisting of leaf mottling in a small proportion of apical leaves (Fig. 7). Nonetheless, these 6 transgenic plants grew well, flowered, and set fruits and seeds similar to the other transgenic plants.

The extent of viral multiplication was evaluated by double-antibody sandwich ELISA (DAS-ELISA) and

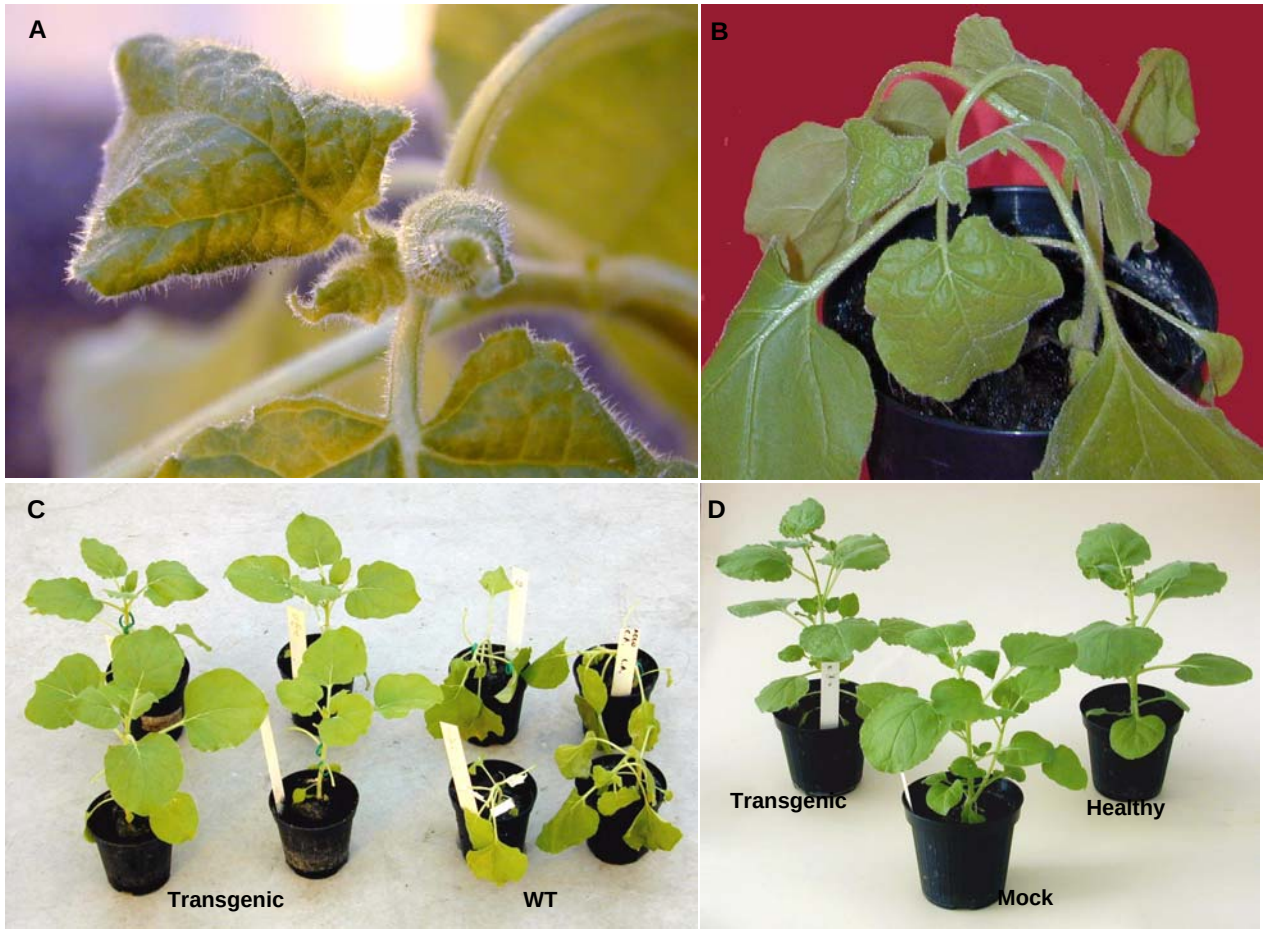


**Figure 5**
PPV inoculated *ihprolC-PP197* transgenic and untransformed control plants. Panel A. Infected, untransformed control plant showing curling of the apical leaves 7 days after inoculation. Panel B. Severe wilting in infected untransformed control plants 7 days after inoculation. Panel C. Transgenic inoculated plants (4 transgenic plants on the left) and untrasformed control plants (4 control plants on the right) 7 days after inoculation. Panel D. Transgenic inoculated (7 days after inoculation), mock-inoculated transgenic and healthy wild-type plants of same age.

immunocapture-rtPCR (IC-RT-PCR). Seven days after inoculation, samples from inoculated basal leaves (half leaves) and from non-inoculated apical leaves of all plants were analyzed. Inoculated basal half-leaves were positive by ELISA in both wild-type and transgenic plants (Table 1). Moreover, the ELISA values were rather similar in wild-type and transgenic plants (Table 1). Thus, neither local viral infection nor replication is prevented in *ihprolC-PP197* transgenic plants. The non-inoculated half leaves from 25 transgenic plants were also analysed by DAS-ELISA. In 21 of 25 plants tested, the ELISA values indicated that they were free of virus (below detection limits) (data not shown).

The systemic spreading and replication of PPV was evaluated by ELISA performed on apical non-inoculated leaves (Table 2). At 7 days, apical (non-inoculated) leaves from the progeny of plant lines 13 and 29 were negative in all transgenic plants. The absorbance values were never higher than the average value plus 3 times the standard deviation measured in leaves of mock-inoculated transgenic and healthy wild type plants. Seven days after inoculation, 10 out of 25 plants from the progeny of plant lines 2, 8 and 9, were positive, while the other 15 plants gave values below detection levels (Table 2).

In order to evaluate whether PPV was able to overcome the systemic resistance conferred by the *ihprolC-PP197*

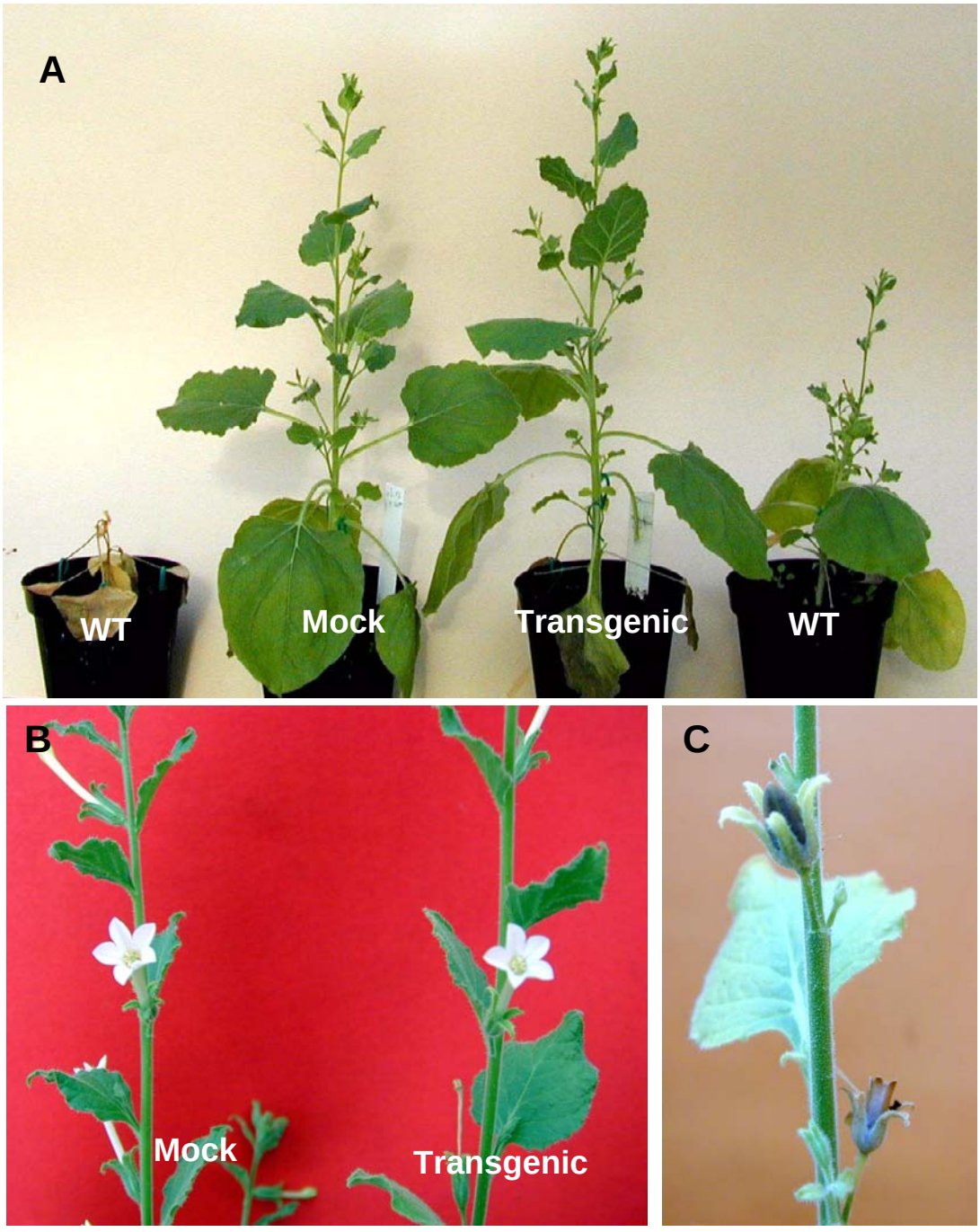


**Figure 6**
PPV inoculated *ihprolC-PP197* transgenic and untransformed control plants 15 days after inoculation. Panel A. From left to right, infected (dead) untrasformed control plant, mock-inoculated transgenic plant, transgenic inoculated plant, infected (severely sick) untransformed control plant. Panel B. Flowers from transgenic PPV inoculated (right) and from mock-inoculated transgenic (left) plants. Panel C. Capsules of PPV inoculated transgenic plant.

**Figure 7**
Symptoms in PPV inoculated transgenic plant. Leaf mottling observed in a transgenic plant 25 days after PPV inoculation.

gene during later phases of plant growth, ELISA was again performed 6 weeks after inoculation (Table 2). All transgenic plants were negative, with the exception of the 6 transgenic plants from the progeny of plant lines 8 and 29 that showed mild symptoms of PPV infection. These plants contained PPV in apical leaves (Table 2). It is noteworthy to stress that all the other symptomless plants that were ELISA positive one week after inoculation, were negative 6 weeks after inoculation.

In order to validate the ELISA analysis, 30 samples from ELISA-negative, asymptomatic plants were randomly chosen and analysed by IC-RT-PCR, a technique that is at least 1000-fold more sensitive than ELISA in detecting PPV [29]. All samples were also negative by IC-RT-PCR, confirming the absence of the virus and validating the ELISA assay.

### *Transitivity of siRNAs in PPV inoculated* Nicotiana benthamiana *plants transgenic for the* ihprolC-PP197 *gene*

SiRNAs were analysed in PPV inoculated plants. Northern blot analysis was performed using probes homologous either to the 197 bp region used in the hairpin construct (Fig. 8, panel A) or to PPV sequences located between 1000 and 1500 bases at the 3' of the 197 bases used in the hairpin construct (Fig. 8, panel B). siRNAs homologous to the 197 bp region of the hairpin construct were detected in inoculated transgenic plants both 2 days after inoculation (Fig. 8, panel A, lanes 2 and 3) and 42 days after inoculation (Fig. 8, panel A, lane 4). Two classes of siRNAs were found: namely, 23 and 25 nt long species (Fig. 8, panel A, lanes 2–4). siRNAs were absent in inoculated wild type controls collected 2 days after inoculation (Fig. 8, panel A, lane1). When the same blot was hybridised to a probe derived from viral sequences located between 1000 and 1500 bases at the 3' of the 197 bases used in the hairpin construct, siRNAs were detected in inoculated transgenic plants 42 days after inoculation (Fig. 8, panel B, lane 4), but were undetectable in inoculated transgenic plants 2 days after inoculation (Fig. 8, panel B, lanes 2 and 3). However, as different plant lines were tested, it is possible that the absence of transitivity observed 2 days after viral inoculation is dependent from the particular line assayed.

Thus, after viral inoculation, transitivity of siRNAs takes place in *ihprolC-PP197* genetically modified plants. It is noteworthy that the transgenic plant analysed 42 days after inoculation (Fig. 8, panel B, lane 4) was virus free and symptomless (plant line 29, plant number 6).

## Discussion

*Plum pox potyvirus* causes severe economic losses of fruit trees belonging to *Drupaceae*. Sharka, the PPV disease, has affected more than 100 million trees worldwide and is considered the most devastating disease of stone fruit trees in Europe [30]. PPV disease has also caused significant damage in South America, Africa and Asia [31]. Just a few years ago, sharka disease was also detected in North America, which is presently facing damage to selected but important stone fruit species [31].

Damage and economic losses are due to systemic PPV infection and disease [15]. To date, conventional breeding has had only limited success in producing commercial varieties of resistant plants [32]. In contrast, the expression of the gene coding for the PPV coat protein under the control of the 35S CAMV promoter conferred resistance to PPV infection in one of 5 peach plants tested [18]. It is

**Table 1: PPV detection in inoculated basal half leaves after seven days of inoculation. DAS-ELISA absorbance values from *ihproIC-PP197* transgenic and wild type plants are reported.**

| | Lines | | | | |
|---|---|---|---|---|---|
| ***Plant*** | 8 | 13 | 29 | 2 | 9 |
| 1 | 0.178 | 0.461 | 0.201 | 0.273 | 0.128 |
| 2 | 0.230 | 0.120 | 0.260 | 0.100 | 0.171 |
| 3 | 0.344 | 0.304 | 1.023 | 0.148 | 0.102 |
| 4 | 0.562 | 0.166 | 0.371 | 0.110 | 0.122 |
| 5 | 0,143 | 0.300 | 0.230 | 0.110 | 0.195 |
| 6 | 0.358 | 0.157 | 0.912 | | 0.132 |
| 7 | 0.216 | | 2.340 | | 0.426 |
| 8 | 0.216 | | 0.981 | | 0.227 |
| 9 | 0.420 | | | | 0.315 |
| 10 | 0.120 | | | | |
| 11 | 0.365 | | | | |
| **WT** | 0.452 | 0.752 | 0.530 | 0.146 | 0.146 |

Samples with absorbance values higher than 0.097 were considered virus-infected. This threshold was calculated as mean absorbance value of the mock inoculated plants plus 3 times the standard deviation of the mean.

**Table 2: PPV detection in non-inoculated apical leaves one week and six weeks after inoculation. DAS-ELISA absorbance values from *ihproIC-PP197* transgenic and wild type plants are reported.**

| | Lines | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 8 | | 13 | | 29 | | 2 | | 9 | |
| ***Plant*** | 1 week | 6 weeks | 1 week | 6 weeks | 1 week | 6 weeks | 1 week | 6 weeks | 1 week | 6 weeks |
| 1 | 0.160 | 0.360* | 0.061 | 0.002 | 0.012 | 0.002 | 0.056 | 0.001 | 0.097 | 0.023 |
| 2 | 0.129 | 0.080 | 0020 | 0.012 | 0.012 | 0.298* | 0.044 | 0.010 | 0.089 | 0.027 |
| 3 | 0.119 | 0.069 | 0.054 | 0.041 | 0.005 | 0.086 | 0.112 | 0.038 | 0.079 | 0.031 |
| 4 | 0.126 | 0.066 | 0.020 | 0.022 | 0.070 | 0.008 | 0.092 | 0.075 | 0.047 | 0.018 |
| 5 | 0.094 | 0.355* | 0.060 | 0.083 | 0.003 | 0.002 | 0.137 | 0.004 | 0.052 | 0.014 |
| 6 | 0.081 | 0.067 | 0.062 | 0.009 | 0.011 | 0.005 | | | 0.117 | 0.001 |
| 7 | 0.057 | 0.072 | | | 0.006 | 0.424* | | | 0.131 | 0.086 |
| 8 | 0.049 | 0.036 | | | 0.008 | 0.873* | | | 0.104 | 0.047 |
| 9 | 0.080 | 0.032 | | | | | | | 0.387 | 0.021 |
| 10 | 0.084 | 0.082 | | | | | | | | |
| 11 | 0.075 | 0.640* | | | | | | | | |
| WT | 0.720 | | 1.520 | | 0.650 | | 0.450 | | 0.450 | |

Samples with absorbance values higher than 0.097 were considered virus-infected. This threshold was calculated as mean absorbance value of the mock inoculated plants plus 3 times the standard deviation of the mean.* Transgenic plants showing leaf mottling (i.e. mild symptoms of PPV infection).

interesting to note that in the PPV resistant transgenic peach plants, PPV was detected by IC-RT-PCR [18]. However, the presence of a very low level of virus genomes, insufficient to cause damage, might be relevant for sustaining an enduring PTGS-based virus resistance. Recently, dsRNA expressed in bacteria has been shown to prevent PPV infection up to 7 days after spraying [33].

PPV can be transmitted either by aphids in a non-persistent manner or by grafting [15]. Aphids inject PPV into leaf epidermal cells. Subsequently, PPV moves to mesophyll cells to bundle sheath and vascular parenchyma cells. From these cells, PPV then moves to companion cells [17]. From the companion cells, PPV enters into the sieve tubes and then is spread by the phloem transport system [17]. Thus, within the plant, PPV spreading over a long distance takes place via the phloem. Consequently,

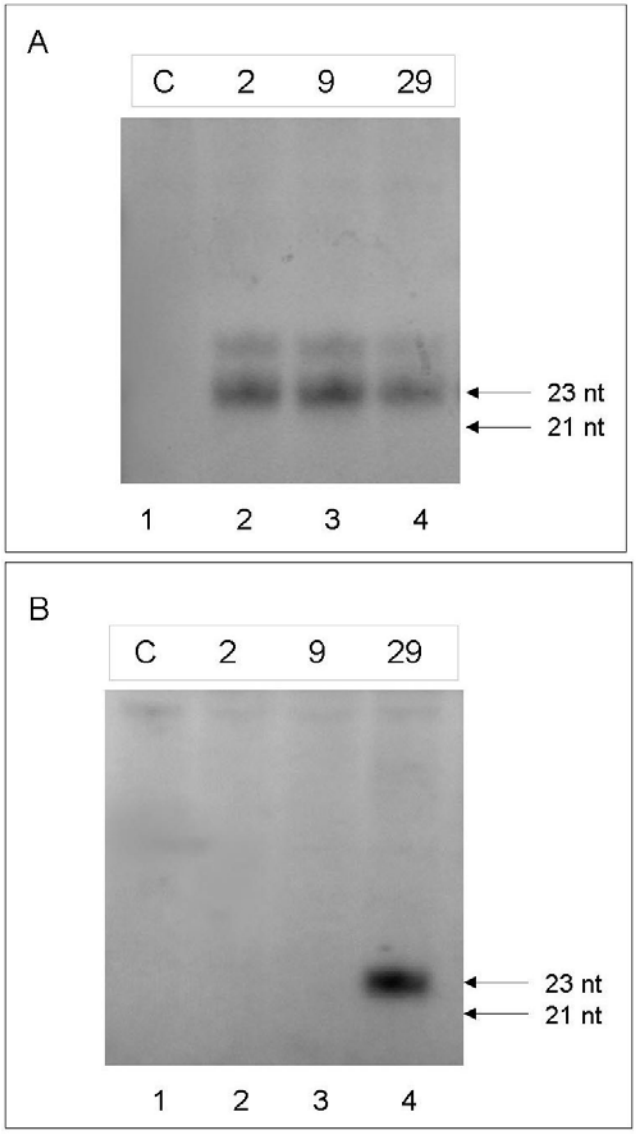


**Figure 8**
RNA gel blot analysis of siRNAs in virus-inoculated *ihprolC-PP197* transgenic plants. Low molecular weight RNA isolated from apical leaves of untransformed control plant (lane 1) and transgenic plants either 2 days (lanes 2 and 3) or 42 days (lane 4) after inoculation were hybridised to hydrolised single stranded $^{32}$P-labeled RNA probe corresponding to the 134–330 region of the PPV genome (Panel A). Panel B shows the same blot hybridised to a riboprobe corresponding to the 1334–1831 region of the PPV genome. 21 and 23 nt-long RNA oligoribonucleotides were used as molecular standards. RNA amount loaded per lane was: Lane 1, 2 and 3, 40 μg; Lane 4, 27 μg.

companion cells and other vascular cell types are placed along the main route of PPV spreading. Expression of antiviral gene constructs in companion cells and other vascular cell types should be apt to interfere with systemic PPV infection.

The progeny of 5 independent transgenic plants expressing the *ihprolC-PP197* hairpin construct were tested for viral resistance. The transgenic progeny of 3 plant lines (lines 13, 2, and 9) were resistant to systemic PPV disease and did not show any symptoms of systemic viral infection during their entire life. In these transgenic plants, apical leaves were PPV negative at 42 days. Thus, systemic spreading of PPV did not take place. It is worthwhile to note that plant line 13 had a single copy of the *ihprolC-PP197* transgene, yet the spreading of PPV was prevented. Additionally, most of the plants belonging to the transgenic progeny of the other 2 plant lines tested (lines 8 and 29) were resistant to systemic disease, and PPV was undetectable in apical leaves. However, 6 out of 19 plants showed mild symptoms (leaf mottling) of PPV disease in a few leaves that were also PPV positive.

In this regard, it is worthwhile to mention that potyviruses encode proteins that act as suppressors of PTGS [34]. In particular, it has been shown that the HC-Pro curtails siRNAs accumulation and inhibits PTGS intracellularly [35]. Current knowledge would suggest that without preventing HC-Pro activity, the viral resistance conferred by PTGS might not be stable. However, HC-Pro does not appear to inhibit systemic signaling of PTGS [36]. During our study, a loss of resistance was never observed. In fact, we have observed in few cases that ELISA values decreased below threshold levels during plant growth (see Table 2).

We have demonstrated that expression of an antiviral-hairpinRNA construct under the control of the *rolC* promoter results in resistance to systemic PPV infection, while local leaf infection is not prevented. This finding is consistent with the fact that the *rolC* promoter does not drive gene expression in non-vascular tissues [21][22][23][24]. In fact, the *rolC* promoter drives vascular-specific expression and is present mainly in companion cells [23], but can also be found in protophloem and other vascular cell types [21][22][23][24]. In this regard, it is worthwhile to stress that the systemic resistance conferred by the *ihprolC-PP197* gene does not prevent virus infection and replication in epidermal and mesophyll cells. PTGS is an adaptative mechanism of defence. Thus, the restricted presence of the viral genome in the inoculated leaves is meant to amplify the PTGS response.

Our data confirm that ihpRNA gene constructs are efficient in eliciting PTGS [12][13]. Moreover, considering that hpRNAs less than 100 nucleotides in length are usually efficient in eliciting RNA silencing [13] and that viral RNA genomes are usually several kb long, it would be

feasible to design several different hpRNA from the sequence of any plant RNA viral genome. In the *ihprolC-PP197*construct, the region of homology to PPV RNA genome spans the AUG initiation codon of the PPV polyprotein. It includes 13 nucleotides of the 5'UTR before the AUG initiation codon and the first 181 bp of the PPV polyprotein coding region, including sequences that are necessary for efficient translation [37] and part of the 18 nucleotide long sequence from base 127 to 145 reported to be required for efficient PPV-host interaction [38]. *IhprolC-PP197* transgenic plants contain siRNAs of 23 and 25 bases homologous to the 197 bases of the PPV genome used in the hairpin construct. It has been previously reported that PTGS correlates with the presence of siRNAs of 2 size classes [9]. Virus inoculated transgenic plants show siRNAs homologous to regions of the viral genome located at the 3' of the sequence used in the hairpin construct. Transitivity of RNA interference involves that the RNA targeted by PTGS functions as template for 5' to 3' synthesis of new dsRNA. Processing of these dsRNA by Dicer RNAse will produce secondary siRNAs. PTGS is an adaptative mechanism of defense. The viral genome is both a target for RNA degradation and a template for PTGS amplification. Thus, resistance results from the interplay of host mechanisms and virus inoculum and replication.

The *ihprolC-PP197* gene, or similar constructs, could be used for engineering resistance to systemic PPV disease in plants belonging to the *Drupaceae*. Moreover, considering that the PTGS state can be transmitted by grafting from a silenced stock to a non-silenced scion [8], it will be interesting to test whether transgenic *ihprolC-PP197* rootstocks will be able to confer resistance to a grafted non-transgenic scion. It is worthwhile to stress that the *ihprolC-PP197* construct does not code for a protein. Moreover PTGS does not appear to alter the transcriptome except for the target RNA [39]. Although the *rolC* promoter is bound to drive expression also in the vascular tissue of the transgenic fruits, fruits should not be modified in their transcriptome, but only in their resistance to PPV.

## Conclusions

Resistance to systemic PPV infection and disease can be efficiently conferred to transgenic plants by using hpRNA constructs expressed in cells of the phloem vascular tissue. The *ihprolC-PP197* gene combines hpRNA technology with vascular specific expression and confers PPV systemic resistance. Local viral infection and replication are not prevented. Thus, in *ihprolC-PP197* transgenic plants, PPV can infect and replicate in epidermal and mesophyll cells, while systemic spreading of the virus is either completely prevented or severely curtailed. Disease symptoms were either completely absent or, in a few plants, consisted of mild mottling in a small number of leaves.

## Methods

### *Gene constructs, recombinant plasmid vectors, and bacterial strains*

A DNA sequence homologous to 197 bases of the viral genome of PPV strain D was synthesised *in vitro* using Taq polymerase and appropriately designed oligonucleotides. The sequence spans the viral genome from base 134 to base 330 (accession number X16415) and, consequently, it includes 13 bases upstream and 181 bases downstream from the AUG initiation codon of the PPV polyprotein. Two 197 bp DNA fragments homologous to the PPV genome were placed in inverted orientation downstream from the 1182 bp DNA fragment containing the promoter of the *RolC* gene of *Agrobacterium rhizogenes* strain A4. An 87 bp DNA fragment containing the 85 base-long intron of the *rolA* gene of *Agrobacterium rhizogenes* was inserted between the two virus-derived sequences (Fig. 1). A 251 bp DNA fragment containing the termination and polyadenylation sequences of the *RolC* gene was placed downstream of the second virus-derived sequence. The resulting gene, termed *ihprolC-PP197*, was subcloned in the T-DNA region of a derivative of pBin19 binary vector [40]. The recombinant pBin19 vector was used to transform *Agrobacterium tumefaciens* strain LBA4404 as described by Shen and Forde [41].

### *Tobacco leaf disc infection and regeneration of transgenic plants*

*Nicotiana benthamiana* plants were cultured on MS medium in Magenta boxes and grown in a growth chamber at 20°C under a 16 h photoperiod. Fully expanded leaves were removed and cut into 1.5 $cm^2$ discs. Leaf pieces were dipped for 20 min in a bacterial suspension. The leaf explants were then removed from the solution and placed upside down on MS medium supplemented with 30 g/l saccharose, 1.0 mg/l 6-benzylaminopurine, 0.1 mg/l α-naphtalene acetic acid, and 7 g/l plant agar. After two days, leaf explants were transferred to fresh MS medium containing 30 g/l saccharose, 1.0 mg/l 6-benzylaminopurine, 0.1 mg/l α-naphtalene acetic acid, 100 mg/l kanamycin, 500 mg/l cefotaxime, and 7 g/l agar. When elongated, putative transgenic shoots were excised from the calli and cultured in Magenta boxes on MS medium supplemented with kanamycin (100 mg/l) without growth regulators to induce rooting. Rooted plantlets were subsequently transferred to soil and grown to maturity in a greenhouse.

### *Plant culture conditions*

*Nicotiana benthamiana* plants were grown in sterile potting mixture in a greenhouse at 24–21°C (day and night temperatures, respectively) with a photoperiod of 14 hours. Independent transformation events (T0) were characterised for their transgenic state and backcrossed with wild-type *N. benthamiana* plants. The T1 progeny of each

primary trasformant were tested for kanamycin resistance and resistance to PPV.

### *Virus source and plant inoculation*

The original PPV isolate, a non-aphid transmissible D-serotype, was supplied by DSMZ Plant Virus Collection (PV0001) and maintained by passage on *N. benthamiana*. The viral inoculum was prepared by grinding 0.5 g of symptomatic *N. benthamiana* leaves in a precooled mortar in presence of 10 ml of 0.05 M potassium phosphate buffer, pH 7.2. Transgenic T1 plants and wild-type controls were inoculated at the stage of 6–7 true leaves. The third, fourth, and fifth leaves were dusted with sterile Celite® and mechanically inoculated on one half of each leaf. Plants were monitored daily by visual inspection.

### *DAS-ELISA and IC-RT-PCR*

PPV-inoculated, transgenic progeny (T1) plants from 5 independent transformation events were tested for virus multiplication by double-antibody sandwich (DAS) ELISA [42] and by immunocapture-RT-PCR (IC-RT-PCR) [43]. Samples from inoculated basal half-leaves and apical non-inoculated leaves were collected from each plant 7 days after inoculation. Samples from apical leaves were also collected 42 days after inoculation. Leaf tissues were ground (1:5 w/v) in extraction buffer and clarified by centrifugation. The supernatant was then used in the tests. Reagents for ELISA (polyclonal antibody and alkaline phosphatase-conjugated polyclonal antibody) were supplied by LOEWE Biochemica GmbH (Germany). Absorbance values at 405 nm were determined using a Microplate Reader 550 (Biorad). Immunocapture-RT-PCR detection of PPV was carried out on the same clarified supernatant used for DAS-ELISA tests and with the same polyclonal antibody supplied in the ELISA kit.

### *Southern blot and PCR analysis*

Genomic DNA was extracted from 0.5–1 g of frozen leaves using the Nucleon PhytoPure system (Amersham Biosciences) according to the manufacturer's instructions. DNA (15 μg) was digested with 70 Units of either *Hind*III or *Dra*I, electrophoresed on a 0.7% agarose gel at 4.5 V $cm^{-1}$, and transferred to a nylon membrane (Hybond N, Amersham Biosciences). The membrane was hybridised with 100 ng of fluorescein-labeled probe prepared using the Random prime labeling module (Amersham Biosciences). Detection was performed with anti-fluorescein conjugated alkaline phosphatase and the chemiluminescent alkaline phosphatase CDP-Star substrate (Amersham Biosciences) according to the manufacturer's instructions. Membranes were exposed for 1 h using Kodak XAR-5 film.

PCR analysis was performed using 100 ng of genomic DNA and the following pairs of primers: one designed in the *rolC* promoter region (5'TGAGATTCCATAGACCACAAACCACC3'; 5'GTTAACAAAGTAGGAAACAGGT3') and the other in the *nptII* coding region (5'CAGAGTCCCGCTCAGAAGAACTCGTCA3'; 5'GGAAGGGACTGGCTGCTATTGGGCGAA3').

### *Small RNA (siRNA) analysis*

Small RNA analysis was performed following the protocol described by Hamilton and Baulcombe [3] with slight modifications. Total RNA was extracted from young leaves with TRIZOL (Invitrogen). The RNA pellet was dissolved in 0.5 ml of sterile DEPC-treated water, heated at 65°C for 5 min, and placed on ice. PEG-8000 (5% final concentration) and NaCl (0.5 M final concentration) were then added. The RNA preparation was then kept for 30 min on ice and high molecular weight nucleic acids were pelleted by centrifugation at 10,000 × g for 10 min. The supernatant containing small RNAs was recovered and further purified by using a RNA/DNA purification kit (Qiagen) to remove contaminants such as proteins, polysaccharides, and carbohydrates. The low molecular weight RNAs were precipitated by adding 3 volumes of ethanol and 1/10 volume of sodium-acetate pH 5.2.

Small RNAs (usually 40 μg per lane) were separated on a 15% polyacrylamide-7 M urea gels and transferred to a Hybond $N^+$ membrane (Amersham Biosciences) by electroblotting for 1 hour at 100 V in 0.5X TBE. The membrane was placed for 10 min on three layers of Whatman 3 MM paper dipped in 20X SSC and then UV cross-linked. Blots were probed with a single stranded RNA corresponding to the region from 134 to 330 of the viral genome obtained by *in vitro* transcription with the Riboprobe *in vitro* transcription system (Promega) using either T7 or T3 polymerase and ($\alpha$-$^{32}$P)-CTP. The probe was hydrolyzed with 300 μl of alkaline buffer (80 mM sodium bicarbonate, 120 mM sodium carbonate) at 60°C for 2.5 hours. The time of hydrolysis was calculated according to the formula reported by Hamilton and Baulcombe [3]. The probe was then neutralised with 20 μl of 3 M sodium acetate, pH 5.2. The membrane was prehybridised at 40°C for 1 hour and, after the addition of the labeled probe, blots were hybridised overnight at the same temperature. The membrane was rinsed twice in 2X SSC/0.2% SDS at 50°C for 10 min and exposed to Kodak X-AR5 film. Oligoribonucleotides of 23 and 21 nt were used as molecular markers. Their sequences were: 5'AAUUGGCAAAGGCCUGGACCCGU3'; 5'AAGGUCCAGGCCUUUGCCAAU3'; 5'AAACGGGUCCAGGCCUUUGCCAA3'; 5'AAAUUGGCAAAGGCCUGGACC3' (Dharmacon). For the transitivity anlysis of siRNA, the probe corresponds to the 1334–1831 region of the PPV genome.

### *Northern blot analysis*

Total RNA was isolated with TRIZOL (Invitrogen) and 10–12 μg of total RNA were separated on 1% agarose-formaldehyde denaturing gels. The gel was blotted on a Hybond $N^+$ membrane (Amersham Biosciences) in 10X SSC. RNA markers were stained with 0.1% toluidine blue in 20% ethanol. The DNA probe, corresponding to the 484 bp-long sequence at the 3' end of the construct, was labeled with ($\alpha$-$^{32}$P)-dCTP using "Ready to go DNA labeling beads (-dCTP)" (Amersham Biosciences). Unincorporated nucleotides were removed by using Probe G-50 micro columns (Amersham Biosciences). The membranes were hybridised overnight at 42°C in ULTRAhyb (Ambion). The membranes were washed 2 times in 2X SSC/0.1% SDS and 2 times in 0.1X SSC/0.1% SDS at 42°C. Autoradiography was then performed using Kodak X-AR5 film.

## Authors' contribution

TP constructed the transgene and supervised the molecular analysis. BM performed the molecular analysis. LA transformed *Nicotiana benthamiana* plants. AS designed the transgene, coordinated the experiments and drafted the manuscript. AP evaluated the response to PPV inoculation and performed the viral detection analysis. All authors read and approved the final manuscript.

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