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Plum Pox Virus Coat Protein Gene Intron-hairpin-RNA (ihpRNA) Constructs Provide Resistance to Plum Pox Virus in *Nicotiana benthamiana* and *Prunus domestica*

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Abstract. **Constructs with self-complementary sequences separated by an intron produce "hairpin" RNA [intron-hairpin-RNA (ihpRNA)] structures that efficiently elicit posttranscriptional gene silencing (PTGS). In the current study, the authors use this technology to confer resistance to plum pox virus (PPV) in herbaceous and woody perennial plants by silencing the PPV–coat protein (*CP*) gene. The authors confirmed the high capacity of ihpRNA constructs for inducing RNA silencing in *Nicotiana benthamiana* Domin., as more than 75% of the transformants displayed PTGS as evaluated by specific small interfering RNA (siRNA) production. The authors demonstrated that ihpRNA constructs provided PPV resistance, and they found a correlation between the length of the *PPV* sequence introduced in the ihpRNA constructs and the frequency of transgenic-resistant plants. Plants transformed with the full-length sequence produced a higher percentage of resistant lines. The authors further demonstrated for the first time that ihpRNA technology is applicable to a woody perennial species. A transgenic plum (*Prunus domestica* L.) *PPV-CP* ihpRNA line showed gene silencing characteristics (hypermethylation of the transgene sequence and specific siRNA production) and resistance to PPV infection 16 months after inoculation.**

Sharka or plum pox, incited by plum pox virus (PPV), is a major disease of stone fruit (*Prunus* L.) responsible for extensive economic losses (Lopez-Moya et al., 2000; Nemeth, 1994). Plum pox virus was first described in Bulgaria in the early 1930s (Atanassov, 1932). It has since spread throughout most of Europe and the Mediterranean region (Llacer et al., 1986; Louro and Corvo, 1986; Mazyad et al., 1992; Refatti et al., 1985; Roy and Smith, 1994). It has also been reported in Chile (Rosales et al., 1998 and in Canada (Thompson et al., 2001), and in the United States in Pennsylvania (Levy et al., 2000), New York (Cornell University, 2006), and Michigan (U.S. Department of Agriculture–Animal and Plant Health Inspection Service, 2006). Movements of nursery stock and infected budwood (Nemeth, 1994) contribute to the long-distance spread of the disease. Plum pox virus is naturally transmitted by a number of aphid species [e.g., *Aphis spiraecola* Patch and *Myzus persicae* (Sulzer)] in a nonpersistent manner (Kassanis and Sutic, 1965; Kunze and Krczal, 1971). There are no effective barriers to stop the natural spread of the disease. The control of PPV remains mostly preventive, through early detection using biological, serological, and molecular methods (Asensio et al., 1994; Desvignes, 1976; Schneider et al., 2004; Varveri et al., 1987; Wetzel et al., 1992). Control through natural host plant resistance has been sought with only

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limited success, and few highly resistant cultivars have been produced (Hartmann, 1998; Sutic and Rankovic, 1981). Cultivars tolerant to sharka provide relief to growers, but tolerant cultivars allow the disease to build up and spread (Kegler et al., 1998).

A promising approach to developing PPV-resistant cultivars is through the application of pathogen-derived resistance (PDR) (Sanford and Johnston, 1985). This technology was first demonstrated by the expression of the coat protein (*CP*) gene of tobacco mosaic virus (TMV) in transgenic tobacco plants that conferred resistance to TMV (Powell–Abel et al., 1986). Since this first report, it has been shown that plants containing *CP* gene sequences can be highly resistant to potyvirus infection (Fitch et al., 1992; Jan et al., 1999; Lindbo and Dougherty, 1992; Scorza et al., 1994; Smith et al., 1995; Sonoda et al., 1999). During the last several years many research laboratories have begun to unravel the mechanism underlying this resistance phenotype. These studies have provided evidence that PDR in many cases is based on RNA silencing, named posttranscriptional gene silencing (PTGS) in plants and RNA interference (RNAi) in animal systems (Hannon, 2002; Kooter et al., 1999; Matzke et al., 2001; Vaucheret et al., 2001; Waterhouse et al., 2001). RNA silencing leads to the degradation of homologous messenger RNAs. In plants, RNA silencing not only participates in the regulation of endogenous gene expression in developmental processes, but it also serves as a component of adaptive protection against mobile genetic elements, such as transposons and viruses (Voinnet, 2005). A key component of the pathway is known as small interfering RNA (siRNA) (Hamilton and Baulcombe, 1999), which is derived from double-stranded RNA (dsRNA), which seems to play a central role in triggering sequence-specific RNA degradation. These siRNAs, corresponding to both sense and antisense strands, guide a multisubunit ribonuclease, the RNA-induced silencing complex, and ensure that it specifically degrades RNAs that share sequence similarity with the dsRNA. Posttranscriptional gene silencing has been demonstrated in herbaceous species, but we know of only one report of functional PTGS in a woody perennial (*P. domestica*) for the development of virus resistance (Hily et al., 2005; Scorza et al., 2001). In this case, resistance was not produced by an intron-hairpin-RNA (ihpRNA) vector, but rather PTGS developed as a result of peculiarities of the insertion event (Scorza et al., 2001).

It has been shown that the expression of self-complementary hairpin-RNA (hpRNA) constructs induces a high level of PTGS in transgenic plants (Wesley et al., 2001). The presence of an intron in between the two complementary regions enhances silencing efficiency (Smith et al., 2000). Pandolfini et al. (2003) showed that the expression of ihpRNA containing a *PPV* sequence, conferred systemic resistance to PPV but did not prevent local infection when introduced into *Nicotiana benthamiana* under the control of the *rolC* promoter. Di Nicola–Negri et al. (2005) reported that more than 90% of transgenic *N. benthamiana* lines were resistant to the virus when engineered with hairpin constructs using *PPV-P1* and *PPV-Hc-Pro* gene sequences under the *35S*-cauliflower mosaic virus promoter.

To apply ihpRNA technology efficiently and effectively for the production of functional PTGS in woody perennials, in this study we tested the effects of different lengths of *PPV-CP* dsRNA and different promoters on the efficiency of inducing PTGS and the resulting resistance to PPV in *N. benthamiana*. The most efficient ihpRNA construct (B14) contained the full length of the targeted gene under the *35S* promoter. We also confirmed the effectiveness of this technology for initiating resistance to PPV in a woody perennial fruit tree, *P. domestica*, using the B14 ihpRNA construct.

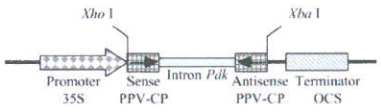
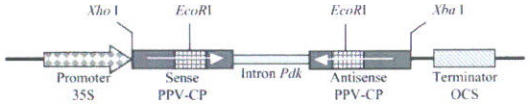
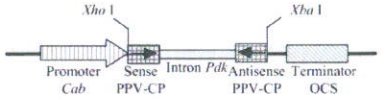
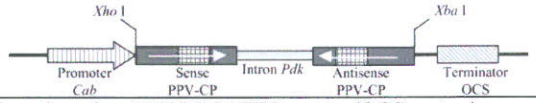
Materials and Methods

Gene constructs, recombinant plasmid vectors, and bacterial strains. The four plasmid vectors used in this study were derived from the pHELLSGATE8 cloning vector (Wesley et al., 2001) (GenBank accession no. AJ311874). The transcribed regions of the ihpRNA-B1, ihpRNA-B14, ihpRNA-E2, and ihpRNA-E10 (Table 1) constructs were composed of two DNA sequences derived from the *PPV-CP* gene. They were placed in sense and antisense orientations to produce self-complementary dsRNA structures. For B1 and E2 constructs, sequences consisted of 213 bases (from 9027–9239, GenBank accession no. X16415) of the *PPV-CP* gene. This fragment is highly conserved among seven PPV sequences analyzed (GenBank reference nos. X81083, Q01681, U27652, AJ000340, M92280, AJ243957, and X16415). For B14 and E10 constructs, the full length [1 kilobase (kb)] of the *PPV-CP,* as previously described by Scorza et al. (1994), was used. Both *PPV-CP* DNA sequences were synthesized in vitro from plasmid pGA-GG-PPV-CP33 (Scorza et al., 1994). Different restriction enzyme sites were added on each end of the primers for cloning purposes. The *Xho*I site was added to the 5′ end of the sense sequence, whereas *Hin*dIII was added to the antisense sequence. *Asp*718 and *Xba*I sites were added to the 3′ end for the sense and antisense sequences respectively.

Plum pox virus DNA sequences in B1 and B14 constructs were under the control of the *35S* promoter derived from the pHELLSGATE8 vector whereas E2 and E10 hairpin constructs were driven by a *Cab* (chlorophyll a/b binding protein) promoter derived from plasmid pCAPCab19smaB (GenBank accession no. EF127291). The *Cab* promoter was cloned from peach [*Prunus persica* (L.) Batsch. cv. Suncrest] by genome walking and was found to be light responsive and specific to photosynthesizing tissue in its expression (data not presented). The sequence was then subcloned in pSTBlue-1 (Invitrogen, Carlsbad, CA) before substituting for the *35S* promoter from B1 and B14 to produce the two independent constructs E2 and E10.

Transformation systems and regeneration of transgenic plants. *Nicotiana benthamiana* seeds were sterilized and plants were cultured on Murashige and Skoog (MS) salts and vitamins medium (Murashige and Skoog, 1962) in GA-7 Magenta boxes (Sigma-Aldrich Co., St. Louis), and grown at 21 °C under a 16-h photoperiod. *Agrobacterium tumefaciens* (Smith & Towns.) Conn. strain GV3101 containing the hairpin constructs (Table 1) were grown overnight in liquid Luria-Bertani medium containing 50 mg·L^{-1} kanamycin (kan) (Agri-bio, North Miami, FL). Half-mature *N. benthamiana* leaves were removed and sliced in the overnight *A. tumefaciens* suspension. After a 5-min soak in the suspension, leaves were blotted with filter paper and placed on MS salts and vitamins medium supplemented with 25 g·L^{-1} sucrose, 6.5 g·L^{-1} agar, 5 μM benzyladenine for a 2 to 3-d cocultivation. They were then transferred to the same medium with the addition of 100 mg·L^{-1} kan and 400 mg·L^{-1} Timentin (tim; Agri-bio). After 3 weeks, putative transgenic shoots were excised and placed on

Table 1. Schematic drawing of intron-hairpin-RNA constructs carrying the plum pox virus–coat protein (*PPV-CP*) gene.

Name	Gene construct	Resistant T1 plants (%)
B1		39 (N = 44)
B14		73 (N = 37)
E2		15 (N = 27)
E10		68 (N = 22)

All constructs are based on the pHELLSGATE8 vector (OCS, octopine synthase; *Pdk,* pyruvate orthophosphate dikinase intron). B1 and E2 carry the 213 bp of the *PPV-CP* sequence (grid pattern) driven by the cauliflower mosaic virus (CaMV) (*35S*) promoter (solid diamond) or by the *Cab* (chlorophyll a/b protein) promoter (vertical stripes). B14 and E10 carry the full length of *PPV-CP* (1 kilobase), which contains the 213-bp sequence (grid pattern) under either promoter. Indicated are the percentages of *Nicotiana benthamiana* plants resistant to plum pox virus (PPV) 60 d postinoculation (dpi), with N equal to the number of plants tested.

rooting medium, which consisted of half-strength MS salts and vitamins, 20 $g \cdot L^{-1}$ sucrose, 6.5 $g \cdot L^{-1}$ agar, 1 μM indolebutyric acid, 400 $mg \cdot L^{-1}$ tim, and 100 $mg \cdot L^{-1}$ kan. Rooted plantlets (T_0) were transferred to soil and grown for 2 weeks in an environmental chamber (23 °C, 16-h photoperiod, 70% relative humidity). They were tested for the presence of the *neomycin phosphotransferase* (*NPT*II) and *PPV-CP* transgenes then transferred to a greenhouse. T_0 plants were self-pollinated to obtain T_1 progeny, which were germinated and selected on 100 $mg \cdot L^{-1}$ kan. Polymerase chain reaction (PCR) analysis, using primers specific to the *PPV-CP* gene, of a subset of seedlings surviving under kan selection (17 plants) indicated a selection efficiency of 94%.

Prunus domestica transformation using seeds of 'Stanley' followed the protocol described by Padilla et al. (2003) using *A. tumefaciens* LBA4404 carrying the ihpRNA-B14 or the -E2 constructs. Before transfer to the greenhouse, plants were tested by PCR for the *NPT*II and *PPV-CP* transgenes, as described by Scorza et al. (1994).

Virus source, plant inoculation, and double antibody sandwich ELISA (DAS-ELISA). To investigate virus resistance, *N. benthamiana* T_1 lines were selected based on siRNA production of T_0 parental lines and inoculated with PPV-PENN 3 (GenBank accession no. DQ465242), a D type of PPV that was isolated from infected *Prunus* in Pennsylvania (Levy et al., 2000). For each of the 16 T_1 lines inoculated, 3 to 11 individual transgenic plants were tested.

Transgenic *P. domestica* was also inoculated with PPV-PENN 3. All inoculation studies were performed under P3 containment conditions at the U.S. Department of Agriculture–Agricultural Research Service, Foreign Disease and Weed Science Research Unit, Ft. Detrick, MD, under Animal and Plant Health Inspection Service (APHIS) permit no. 15807. Transgenic *N. benthamiana* and control plants [pea (*Pisum sativum* L.)] were mechanically inoculated. 'Colmo' pea leaves infected by PPV-PENN 3 were ground in a 50-mM sodium phosphate buffer with 20 mM sodium diethyldithiocarbamate trihydrate and 5 mM EDTA. The resulting inoculum was then rubbed on the two lowest leaves of test plants that had been lightly dusted with 400-grit carborundum as an abrasive. Plants were then tested for the presence of PPV by evaluating two to three young leaves from each plant. At 10 d postinoculation (dpi), 160 plants were tested; 134 plants were tested at 60 dpi.

Transgenic *P. domestica* and control plants (peach and untransformed *P. domestica*) were inoculated via aphids (*M. persicae*). Aphids were starved for 30 min and then allowed to feed on highly symptomatic leaves of 'Lovell' peach seedlings for virus acquisition. Aphids were then released into cages with test seedlings.

Inoculated plants were placed under natural daylight conditions supplemented with 400-W lamps to provide a 15-h photoperiod. Temperature was maintained at 24 °C.

Plum pox virus infection of test plants was evaluated by visual observation of symptoms and by DAS-ELISA. Leaf samples ranged from 0.25 to 0.5 g and were ground in phosphate-buffered saline-Tween ELISA buffer at a 1:10 ratio (w:v). Following centrifugation for 5 min at 1200 g_n (Beckman GPR centrifuge; Beckman Instruments, Palo Alto, CA), the supernatant was collected and then processed according to the manufacturer's protocol (REAL Kit; Durviz S.L.U., Paterna, Spain). Plants showing ELISA values three times over the background were considered positive. *Prunus domestica* were also tested for the virus using real-time (RT)-PCR (TaqMan; Applied Biosystems, Foster City, CA) technology as described by Schneider et al. (2004).

DNA blot and DNA methylation analysis. Plum DNA extraction, DNA blot analysis, and detection were performed as previously described by Scorza et al. (1994, 2001). For DNA

blotting, 15 µg DNA was digested with *Xba*I (New England Biotechnology, Beverly, MA) or *Xho*I/*Xba*I (New England Biotechnology) according to the manufacturer's instructions and resolved on a 0.8% agarose gel. The full length of the *PPV-CP* gene was used as the probe.

Copy number evaluation in *N. benthamiana* lines was performed on DNA that was digested with *Xba*I enzyme. Methylation status was evaluated as previously described by Hily et al. (2004). Briefly, restriction digestion of 5 µg genomic DNA was carried out overnight at 37 °C, using 1 $U \cdot \mu g^{-1}$ for each enzyme (methylation-sensitive *Sau*3AI and methylation-insensitive *Mbo*I; Gibco BRL, Life Technologies, Gaithersburg, MD) in the presence of 5 mM spermidine in a volume of 200 µL. Quantitative PCR was performed using an ABI Prism 7900HT sequence detection system with 2× SYBR Green PCR Master Mix (Applied Biosystems). The PCR reactions were performed in triplicate for each sample with primers specific to *Sau3*AI restriction sites located at positions 340 and 600 bp in the *PPV-CP* gene (Hily et al., 2004) and *Sau*3AI sites of *Cab* gene. Primers Cab *Sau3*AI fwd (5′ cta tct tgg caa ccc aaa cct 3′), Cab *Sau3*AI rev (5′ gtg gat cca gtc ctt cac caa 3′), Cab nosau fwd 452 (5′ ttg tcc atg ccc aga gca t 3′), and Cab nosau rev 519 (5′ tcc ttc gac aaa tcc cat gag 3′) were designed by Primer Express 2.0 software (Applied Biosystems) and were used as internal controls. The unmethylated *Sau*3AI sites in the *Cab* gene were used as controls to verify activity of the *Sau*3AI restriction enzyme. If the *Sau*3AI sites in the *PPV-CP* transgene were methylated, *Sau*3AI digestion would be inhibited, and the higher the level of methylation, the higher the amplification value of the undigested sequences. Values were quantitatively standardized and take into account the level of digestion. A dissociation curve analysis was performed after each run to screen for non-specific products.

Small interfering RNA analysis. Total RNA was extracted from 0.1 g leaves using the Purescript kit (Gentra Systems, Inc., Minneapolis, MN). The pellet was dissolved in 50 µL 50% formamide. Relative quantification of low-molecular weight RNA was performed on a 1% nondenaturing agarose gel using ethidium bromide staining. Before separation, samples were heat treated at 95 °C for 4 min and placed on ice for 10 min. Then total RNA was separated on a 20% polyacrylamide–7 M urea gel and transferred to a Hybond Nx membrane (Amersham Biosciences, Little Chalfont, UK) by electroblotting at 80 mA for 45 min in a 0.5× Tris-Borate-EDTA (TBE) buffer (Sambrook and Russel, 2001). Nucleic acids were then fixed by ultraviolet (UV) cross-linking, using a UV stratalinker 1800 (Stratagene, Garden Grove, CA).

Hybridization was performed using in vitro synthesized ^{32}P-labeled DNA *PPV-CP* transcripts generated as previously described (Hily et al., 2005). The full sequence (1 kb) of *PPV-CP* was used as template for ihpRNA-B14 and ihpRNA-E10 constructs, and the short-length (213 bp) *PPV-CP* sequence was used for ihpRNA-B1 and ihpRNA-E2 constructs.

Results

Analysis of T_0 transgenic *Nicotiana benthamiana* plants carrying *PPV-CP* ihpRNA constructs and ability to activate gene silencing pathway. Six to 16 *N. benthamiana* lines that tested positive by PCR for *PPV-CP* and *NPTII* genes were obtained from each of the four pHELLSGATE8-based constructs (Table 1). More than half of the lines (59%, 24 of 41 lines tested) contained multiple copies of the transgene (Fig. 1).

Among the 48 transgenic lines obtained, 37 (77%) produced detectable siRNA (Fig. 1), confirming that the ihpRNA constructs used in this study induced a high percentage of transgenic plants displaying PTGS as determined by the presence of

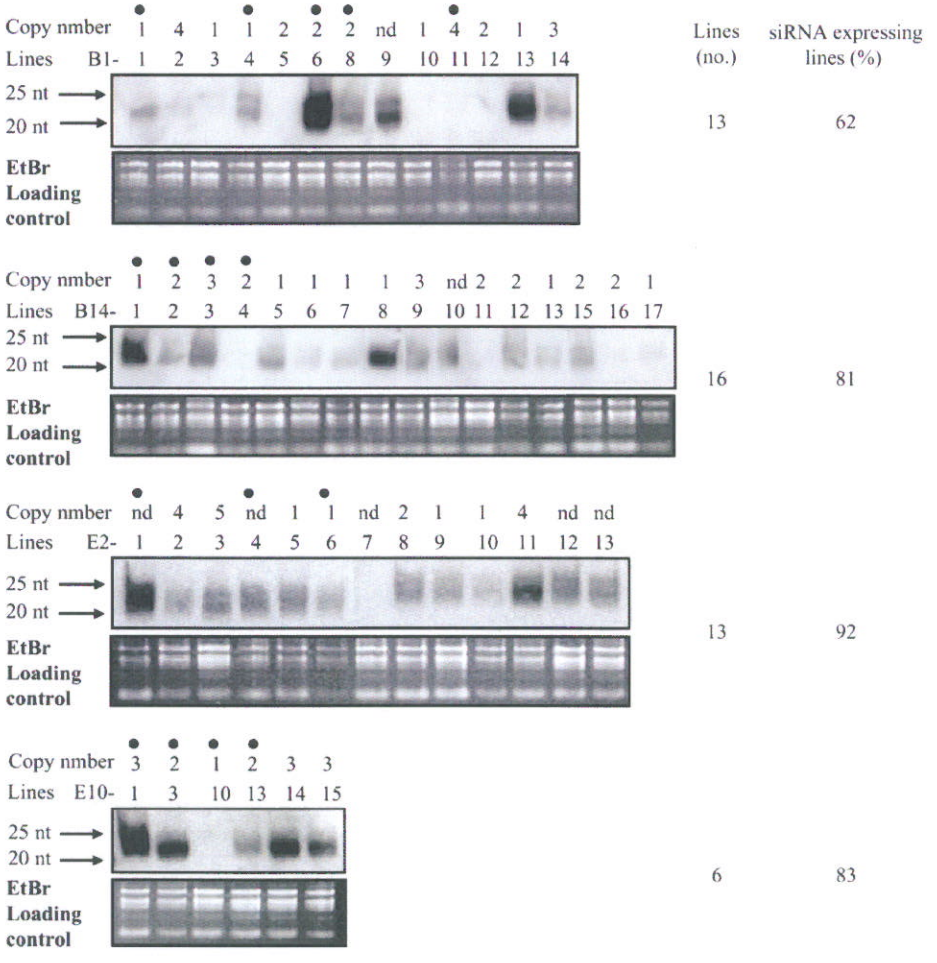


Fig. 1. RNA gel blot for detection of short interfering RNA for plum pox virus–coat protein (*PPV-CP*) transgene. This analysis was performed on noninoculated tissue from all T_0 generation transgenic lines. Arrows at left indicate nucleotide (nt) position markers. Relative quantification was performed on a 1% nondenaturing agarose gel, using ethidium bromide (EtBr) staining. Percentage of small interfering RNA (siRNA)-expressing lines per construct is indicated as well as copy number of the transgene. The designation "•" indicates lines that were inoculated to perform resistance tests.

PPV-CP-specific siRNA, which validated previous work (Wesley et al., 2001). It appeared that more transgenic lines produced siRNA under the *Cab* promoter (90%, 17 of 19; Fig. 1, E2 and E10) than with the constitutive *35S* promoter (69%, 20 of 29 lines; B1 and B14). For each construct tested, variability in siRNA accumulation level was observed: from no siRNA, for example, in lines B1-11, B14-4, E2-7, or E10-10; to high levels, for example, in lines B1-6, B14-1, E2-1, or E10-1 (Fig. 1). DNA blots to determine insert copy number indicated that there was no correlation between copy number and siRNA accumulation (Fig. 1). The length of the transgenic *PPV-CP* sequence did not appear to influence the percentage of lines expressing siRNA or siRNA accumulation. All transgenic *N. benthamiana* lines grew normally, developed normal flowers, and set viable seeds.

Screening for plum pox virus resistance in *Nicotiana benthamiana* T_1 generation. To evaluate the effect of siRNA accumulation on resistance to PPV we selected for resistance tests T_1 progeny of T_0 lines that were not expressing siRNA (B1-11, B14-4, and E10-10), and others that were accumulating varying levels of siRNA from low (B1-1, B14-2, E2-6, E10-13), to moderate (B1-4, B1-8, B14-3, E2-4, E10-3) to high (B1-6, B14-1, E2-1, and E10-1) based upon RNA blots (Fig. 1). At 10 dpi (Fig. 2A), 88% (seven of eight) of the untransformed "wild-type" controls plants were found to be highly positive for PPV by ELISA. A similar percentage of infected plants, 77% (17 of 22), was observed in the T_1 generation of transgenic T_0 lines that were not producing siRNA (lines B1-11, B14-4, and E10-10). When T_0 lines were producing siRNA, the numbers of PPV-resistant T_1 progeny were much higher. Initial results at 10 dpi suggested that there was a positive correlation between parental siRNA levels and PPV resistance of T_1 progeny (Fig. 2A), but at 60 dpi it was apparent that the parental level of siRNA was not critical for progeny resistance with an average overall siRNA producing T_0 lines of 54% resistant (46% infected) T_1 progeny.

Although transgene sequence length did not affect the percentage of T_0 plants that produced siRNA in the progeny of siRNA-expressing lines, the length of the transgene sequence influenced the percentage of resistant plants. Indeed, at 10 dpi, 46% (39 of 84) of the progeny containing the short length of *PPV-CP* (e.g., B1 and E2 lines) were infected whereas only 18% (12 of 68) of the transgenic plants transformed with the full sequence of *PPV-CP* (e.g., B14 and E10 lines) were positive for PPV by ELISA. After 60 dpi, 33% of the progeny of the T_0 plants expressing the short length of *PPV-CP* were found to be resistant whereas 71% of the T_1 plants expressing the full length of the *PPV-CP* sequence were found to be resistant to the virus (Fig. 2B), indicating a positive and stable effect of the full-length sequence on resistance. At 10 dpi, no statistical differences (analysis of variance, $P < 0.05$) in infection percentage were observed between plants transformed with ihpRNA under the control of either the constitutive *35S* promoter or *Cab* promoter, with 66% and 68% of resistant plants respectively. After 60 dpi, plants transformed with *35S* promoter produced a higher percentage (53%) of resistant plants than lines containing the *Cab* promoter (39%; Fig. 2C). In summary, the T_0 lines producing the highest percentage of resistant progeny were those expressing the full-length *PPV-CP* ihpRNA. The *35S* promoter improved resistance of the progeny from T_0 lines expressing siRNA.

Evidence for posttranscriptional gene silencing in resistant transgenic lines. To verify that PTGS was underlying PPV resistance, we investigated the production of siRNA in 16 resistant and sensitive T_1 generation plants from T_0 clones B1-1, B1-4, B1-6, and B1-11 (Fig. 3). Small interfering RNAs were extracted from leaves that were sampled before inoculation. Small interfering RNAs specific to the *PPV-CP* gene were detected in RNA samples isolated from leaf tissue of resistant plants whereas no siRNAs were observed in samples from sensitive plants. These results were further confirmed with samples from nine T_1 plants, three each from T_0 clones B14-1, B14-2, and B14-3 (data not presented). These data indicate that siRNA production was correlated with virus resistance in ihpRNA transgenic lines and that resistant lines underwent RNA silencing.

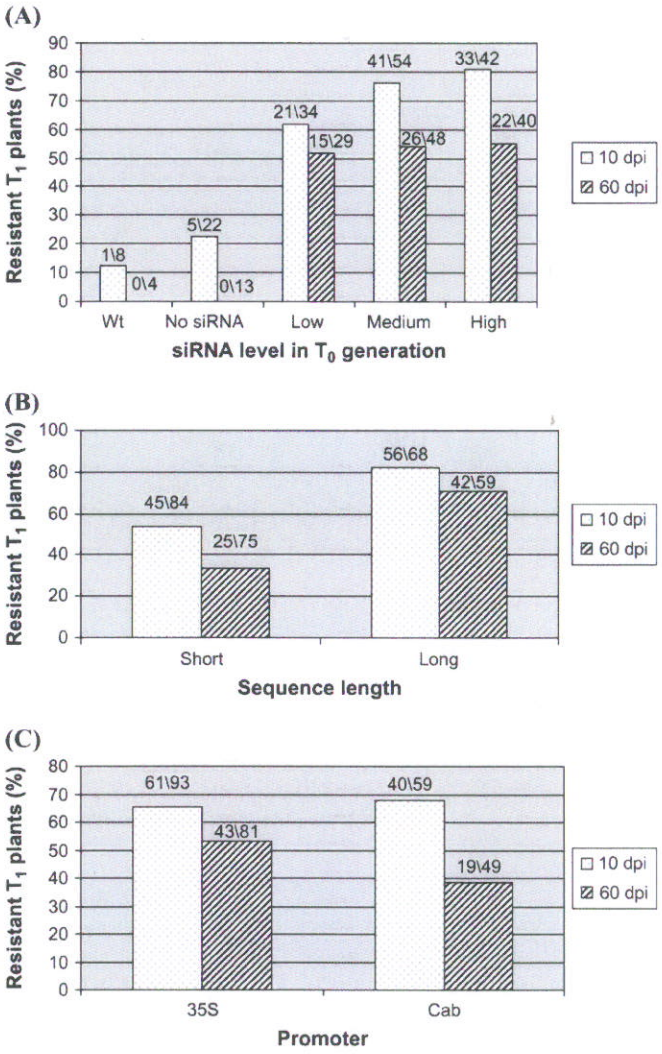


Fig. 2. (**A–C**) Percentage of resistant T_1 *Nicotiana benthamiana* plants to plum pox virus 10 and 60 d postinoculation (dpi). Data include all plum pox virus–coat protein (*PPV-CP*) intron-hairpin-RNA (ihpRNA) constructs tested. (**A**) The influence of parental (T_0) small interfering RNA (siRNA) level on resistance in progeny. (**B**) The influence of *PPV-CP* insert length (full length, B14 and E10 constructs; short length, B1 and E2 constructs). (**C**) The influence of promoter [cauliflower mosaic virus (CaMV) (*35S*) in the B1 and B14 constructs; chlorophyll a/b binding protein (*Cab*) in the E2 and E10 constructs] on resistance in siRNA-expressing lines progeny. Wt, wild type. Number of resistant plants/number of plants tested is indicated.

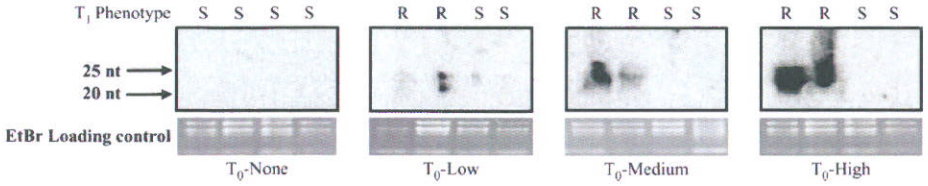


Fig. 3. Small interfering RNA (siRNA) accumulation in intron-hairpin-RNA (ihpRNA) *Nicotiana benthamiana* T_1 progeny from T_0 lines shown in Fig. 1 that produced no siRNA (line B1-11), a low level of siRNA (B1-1), a moderate level of siRNA (B1-4), or a high level of siRNA (B1-6). Leaf tissues from progeny were sampled before virus inoculation. Resistant (R) and sensitive (S) phenotypes were then tested for the presence of plum pox virus–coat protein (*PPV-CP*) transgene-specific siRNA. Relative quantification was performed on a 1% nondenaturing agarose gel using ethidium bromide (EtBr) staining. nt, nucleotide.

Transgenic plums, small interfering RNA production, and resistance to natural plum pox virus infection. A number of transgenic *P. domestica* clones were produced from transformation with the ihpRNA constructs shown in Table 1. One plum clone, developed from the ihpRNAB14 construct containing the full-length *PPV-CP* hairpin and *35S* promoter, which was the most efficient overall in inducing PPV resistance in *N. benthamiana,* was evaluated for PPV resistance. DNA gel blot analysis showed that digestion of genomic DNA from this ihpRNA-B14 plum line with *Xba*I and *Xho*I released a 2.8-kb fragment band corresponding to the expected size of the insert (Fig. 4). To determine the copy number of the insert, plum genomic DNA was digested with *Xba*I and was blotted, and only one clear, intense band was detected (Fig. 4), indicating that the transformed plum line likely harbors a single insert. After 1 month at 24 °C in a controlled environment, DNA was extracted and analyzed for methylation status using real-time quantitative PCR (TaqMan) (Hily et al., 2004). The ihpRNA-B14 plum clone displayed high levels of methylation of the *PPV-CP* transgene at the two *Sau*3AI sites tested: 340 and 660 (Fig. 5). Increased DNA methylation has been shown to be closely associated with PTGS (Guo et al., 1999; Jones et al., 1999; Sonoda et al., 1999). Methylation levels in ihpRNA-B14 plum was higher in comparison with those in C3 [a *PPV-CP* transgenic clone sensitive to PPV (Hily et al., 2004)] and similar to levels reported in C5, a highly resistant *PPV-CP* transgenic clone displaying PTGS (Hily et al., 2004; Scorza et al., 1994, 2001).

The ihpRNA-B14 plum clone constitutively produced a short (21 nt) and a long (25–26 nt) class of siRNA (Fig. 6). The siRNA accumulation levels in the ihpRNA-B14 clone were found to be similar to C5 *P. domestica* (Hily et al., 2005), suggesting that the clone would be highly resistant to PPV. To verify that hypothesis, small rooted plantlets of ihpRNA-B14 plum, 1 month from in vitro culture, were inoculated with PPV by being exposed to viruliferous aphids. Two months after inoculation, all seven plantlets analyzed were symptomless and tested negative for PPV by ELISA (Table 2). Control plants (pea, peach, and untransformed *P. domestica*) under the same conditions produced clear symptoms and were found ELISA positive for PPV. ELISA and quantitative RT-PCR (TaqMan) analyses at 8 and 16 months after inoculation confirmed the 2-month results, with no virus detected. The ihpRNA-E2 construct were also used to transform *P. domestica*. Three clones, obtained from three different explants, were tested with PCR, and amplified a product around 210 bp, corresponding to the short *PPV-CP* sequence, and tested negative for the *A. tumefaciens Vir* gene. Those three ihpRNA-E2 lines also produced the two classes of siRNA present in C5 (Fig. 6B). Evaluation of resistance in these lines is underway.

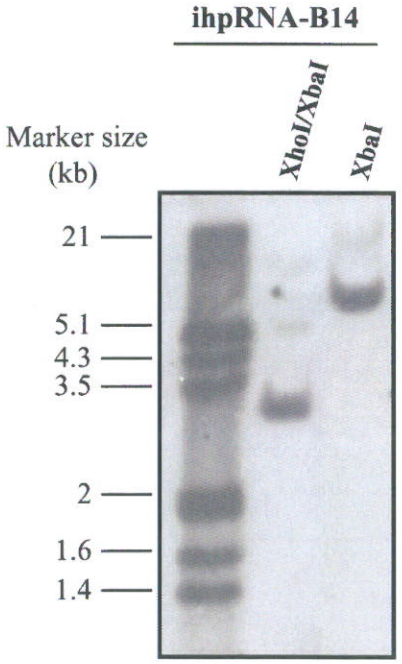


Fig. 4. DNA gel blot analysis of the intron-hairpin-RNA (ihpRNA)-B14 transgenic *Prunus domestica* line. A 2.8 kilobase (kb) band is expected for a single insertion when samples are digested by *Xho*I/*Xba*I. The *Xba*I digestion confirmed insertion in the plum genome (see Table 1 for restriction sites).

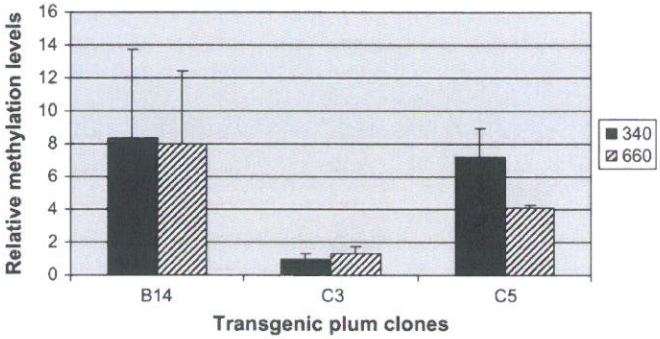


Fig. 5. Relative methylation quantification in the intron-hairpin-RNA (ihpRNA)-B14 transgenic plum line compared with C3 and C5 (Scorza et al., 1994) by polymerase chain reaction (TaqMan; Applied Biosystems, Foster City, CA). The x-axis indicates the clones that were evaluated for methylation at sites *Sau3AI* 340 and *Sau3AI* 660 present in the plum pox virus–coat protein (*PPV-CP*) sequence (Hily et al., 2004). Values represent relative methylation quantification levels of amplification obtained from the threshold cycle number. There is a linear relationship between the amplification value and the amount of "amplifiable" starting material. A greater level of DNA methylation is indicated by a higher amplification value. Values are the average of at least three independent experiments. Error bars indicate SD.

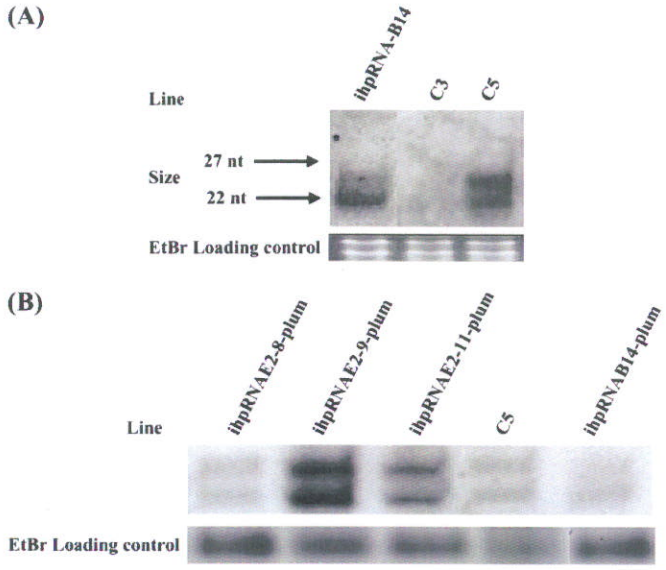


Fig. 6. (**A, B**) Small interfering RNA (siRNA) production in leaves from a transgenic uninoculated *Prunus domestica* containing the intron-hairpin-RNA (ihpRNA)-B14 construct (**A**) and the ihpRNA-E2 construct (**B**). C5 or C3, a resistant and a sensitive transgenic plum, were used as positive and negative controls respectively for siRNA production. Nucleic acid preparations were separated on a 20% denaturing polyacrylamide gel and hybridized with radioactive DNA probes corresponding to both strands of the full-length plum pox virus–coat protein (*PPV-CP*) sequence (Scorza et al., 1994). Positions of the 22 and 27 nucleotides (nt) markers are at the left. Relative quantification was performed on a 1% nondenaturing agarose gel using ethidium bromide (EtBr) staining.

Discussion

The use of ihpRNA in this study is a PDR (Sanford and Johnston, 1985) approach that relies on the production of dsRNA through the transcription of a self-complementary hairpin structure based on viral *CP* sequences that interferes with the virus at the RNA level. This strategy is based on the natural plant virus defense mechanism that functions through RNA silencing and is triggered by dsRNA, an intermediate RNA form in the replication of many plant viruses.

Table 2. Plum pox virus (PPV) detection in transgenic *Prunus domestica* line intron-hairpin-RNA-B14 from leaves 60 d after aphid-mediated PPV inoculation.

	Means values of absorption ± SD			
	B14 plum	Bbyrd plum	Positive	Negative
Plant A	0.017 ± 0.02	0.656 ± 0.06	0.968 ± 0.09	0.004 ± 0
Plant B	–0.002 ± 0	0.190 ± 0.02		
Plant C	–0.001 ± 0.01	0.559 ± 0.08		
Plant D	–0.005 ± 0.01	0.552 ± 0.05		
Plant E	–0.009 ± 0.01			
Plant F	0 ± 0.01			
Plant G	0.013 ± 0.02			

Double antibody sandwich ELISA absorbance values (± SD) from intron-hairpin-RNA-B14 transgenic plums and from susceptible untransformed controls 'Bluebyrd' plum (Bbyrd). Positive and negative controls were provided by and used according to the manufacturer's protocol.

Transformations with ihpRNA constructs containing different promoters driving different lengths of the *CP* gene sequence of PPV resulted in 62% to 92% of transgenic *N. benthamiana* producing siRNA, which is characteristic of PTGS (Fig. 1). Similar percentages were observed in previous work using ihpRNA constructs (Kalantidis et al., 2002; Smith et al., 2000). Di Nicola–Negri et al. (2005) reported that a majority of transgenic *N. benthamiana* lines (38 of 40) were resistant to PPV when engineered with *PPV-P1* and *Hc-Pro* hairpin constructs. This confirms the effectiveness of this technology for initiating a high percentage of transformants displaying RNA silencing. We found a wide range of *PPV-CP* siRNA accumulation levels in our transgenic lines, from no siRNA expression to a high level of accumulation. Our results suggest that *PPV-CP* siRNA accumulation levels are not related to the transgene copy number. A similar observation was made by Chuang and Meyerowitz (2000), who showed that the level of silenced phenotype in AP1 (RNAi) T1 pants was not correlated to the transgene copy number.

The T_1 progeny of 16 independent T_0 transgenic lines transformed with PPV ihpRNA constructs were tested for viral resistance. T_1 progeny from T_0 mother plants that did not constitutively produce siRNA were as susceptible as untransformed controls, with 100% of plants infected 60 dpi. When the T_0 plants expressed siRNA, the progeny displayed variable levels of resistance and acted differentially depending on the construct they were expressing. We observed that resistance to PPV and siRNA accumulation was positively correlated in the progeny. All the resistant plants tested produced siRNA whereas none of the susceptible clones did. We observed after 60 dpi that the T_1 generation of plants transformed with the full length of the *PPV-CP* (1 kb) was significantly more resistant (71%) than plants transformed with a shorter 213-bp length (33%) of the same *PPV-CP* insert (Fig. 2). Similar observations between size of the transgene and resistance were made in transgenic papaya (Pang et al., 1997; Tennant et al., 2001). RNAi has also been reported to depend on the length of dsRNA in plants, nematodes (*Caenorhabditis elegans* Maupas), and fruit flies (*Drosophila melanogaster* Meigen) (Fire et al., 1998; Hammond et al., 2000). Recombinant viruses carrying fragments of 300 to 800 nt derived from the target gene are usually used for efficient triggering of RNAi of a transgene in plants. Silencing appears to be most efficient when sequences of more than 300 bp are used in target ihpRNA constructs. This may indicate that the ability of dsRNA to interfere specifically with virus infection requires not only homology of sequence but also a minimum length of dsRNA. We can draw the same conclusion from our work because both sequences (full and short length of *PPV-CP* strain D) are more than 99% homologous with the virus strain PPV PENN-3 (W. Schneider, pers. comm.).

Different promoters, including *35S* and tissue specific, have been used to drive ihpRNA in plants (Byzova et al., 2004; Helliwell and Waterhouse, 2003). In this report we demonstrated that ihpRNA constructs of the *PPV-CP* gene, under *35S* promoter and a *Cab* promoter cloned from peach, produced resistance to the targeted virus. We found that the *35S* and the *Cab* promoters did not significantly affect the ability of ihpRNA to trigger gene silencing. Nevertheless, *35S*, considered a "strong" and constitutive promoter, increased the percentage of virus-resistant plants in the long term (60 dpi). The effectiveness of this promoter may have been related to the mechanical inoculation protocol that allows a higher titer of

virus to be introduced into leaves compared with natural aphid infection. Nevertheless, under these conditions we did obtain resistant plants using the *Cab* promoter. It would be interesting to evaluate the resistance of plants containing the *Cab* promoter under natural infection, mediated by aphids. In this case we speculate that the percentage of resistant plants may be higher than that obtained under mechanical inoculation as a result of the lower virus inoculation pressure produced by aphids.

In this study we demonstrated the utility of ihpRNA interference technology in a woody perennial tree. Plum clone ihpRNA-B14 displayed the characteristic of PTGS with high levels of DNA methylation specific to the *PPV-CP* transgene (Fig. 5). This clone also produced levels of the two classes of siRNA (Fig. 6) that were similar to those produced by C5, a highly resistant transgenic plum (Hily et al., 2004; Scorza et al., 1994, 2001). Furthermore, we demonstrated that the ihpRNA-B14 plum clone was resistant to aphid-vectored PPV. A number of additional plum lines containing the B14 and E2 constructs are currently being tested for virus resistance. Our results indicate the potential for the use of ihpRNA technology for improving woody perennial species. We suggest that ihpRNA technology will be useful not only for the development of virus resistance but for the improvement of numerous traits, including fruit quality, by using specific promoters driving ihpRNA to control the temporal and spatial expression of genes. The positive function of the peach-derived *Cab* promoter suggests that this promoter may be a useful tool in further Rosaceae genetic engineering.

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