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Engineering Resistance to Multiple *Prunus* Fruit Viruses Through Expression of Chimeric Hairpins

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Abstract. ***Prunus*** **L. fruit production is seriously affected by several predominant viruses. The development of new cultivars resistant to these viruses is challenging but highly desired by breeders and growers. We report a posttranscriptional gene silencing-based approach for engineering multivirus resistance in plants. A single chimeric transgene, *PTRAP6,* was created by the fusion of 400 to 500-base pair (bp) gene fragments from six major *Prunus* fruit viruses, including american plum line pattern virus, peach mosaic virus, plum pox virus (PPV), prune dwarf virus (PDV), prunus necrotic ringspot virus, and tomato ringspot virus (ToRSV). Both strands of *PTRAP6* were found being transcribed as an ≈2.5-kilobp transcript in planta without splicing interruption. To induce gene silencing/virus resistance, we placed two copies of *PTRAP6* in an inverted repeat under the control of the cauliflower mosaic virus 35S promoter and separated by an intron spacer fragment to create *PTRAP6i*. Inoculation of the resulting transgenic *Nicotiana benthamiana* Domin. plants revealed that 12 of 28 R_0 *PTRAP6i* transgenic lines (43%) were resistant to ToRSV ranging from mild symptoms to symptom-free phenotypes. Detailed analysis of two of three highly resistant homozygous R_3 generation lines demonstrated that they were resistant to all three viruses tested, including PDV, PPV, and ToRSV. The remaining three viruses targeted by *PTRAP6i* were either unavailable for this study or were unable to systemically infect *N. benthamiana*. Transgene-wide and -specific small interfering RNA species were detected along with disappearance of transgene transcript in the resistant lines, indicating that posttranscriptional gene silencing underlies the mechanism of resistance. This work presents evidence that *PTRAP6i* is able to confer gene silencing-based resistance to multiple *Prunus* fruit viruses.**

Commercial *Prunus* stone fruit and nut species, including peach [*P. persica* (L.) Batsch], plum (*P. domestica* L. and *P. salicina* Lindl.), cherry (*P. avium* L. and *P. cerasus* L.), apricot (*P. armeniaca* L.), and almond (*P. dulcis* Mill.), are susceptible to a number of viruses, which can cause serious production losses and decreases in product quality. Prune dwarf virus (PDV), prunus necrotic ringspot virus (PNRSV), and tomato ringspot virus (ToRSV) are widespread in production areas worldwide and are very destructive for peach, plum, cherry, and apricot production (Gilmer et al., 1976; Ogawa et al., 1995). PNRSV-infected peach trees were shown to experience up to 55% production loss over a 4-year period and an 82% loss in 1 year of severe winter cold damage (Saunier, 1972). Plum pox virus (PPV), one of the most serious diseases for stone fruit, has devastated stone fruit production in Europe (Kegler et al., 1998; Ravelonandro et al., 2000). The discovery of PPV in the United States, Canada, and Chile (Levy et al., 2000; Rosales et al., 1998; Thompson et al., 2001) presents a potential threat that PPV may spread widely throughout North America and South America and raises a serious concern in containing and eradicating the virus. Viruses endemic to North America, including peach mosaic virus (PMV) and american plum line pattern virus (APLPV), are also known to cause important diseases of stone fruit (Gilmer et al., 1976). The infection by multiple viruses often causes synergistic reactions more devastating than that incited by a single virus. Sour cherry trees (*P. cerasus*) infected by both ToRSV and PNRSV showed a significant reduction in fruit production and tree growth compared with trees infected by either virus alone (Ramsdell et al., 1992). Similarly, trees infected with both PNRSV and PDV develop severe stunting in peach and gummosis in sour cherry (Howell and Eastwell, 2003), resulting in serious yield reduction (Scott et al., 2001). Breeding virus-resistant cultivars has been one of the challenges for stone fruit breeders for decades. With the inherent long juvenility of tree species and the lack of a natural resistant source in germplasm, developing such a trait through conventional breeding process has proved difficult and there are few, if any, virus-resistant stone fruit cultivars.

The concept of pathogen-derived resistance (PDR) put forward by Sanford and Johnson (1985) facilitated the development of a new avenue for improving virus resistance in plants with modern molecular technology. Since resistance against tobacco mosaic virus through PDR was first reported (Abel et al., 1986), the control of a large number of viral diseases in a wide range of species has been achieved (Fitchen and Beachy, 1993; Goldbach et al., 2003; Lomonossoff, 1995). Introduction of either wild-type or mutated structural or nonstructural viral

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genes into host plant species is able to induce, to varying degrees, resistance to their respective viruses. This approach has proven to be effective for conferring virus resistance in many crop plants (Fitchen and Beachy, 1993; Goldbach et al., 2003; Lomonossoff, 1995). Engineering resistance to stone fruit viruses has also been achieved with the PDR approach. Transgenic herbaceous plants expressing PPV coat protein (CP), cytoplasmic inclusion, proteinase 1, and nuclear inclusion b genes and ToRSV CP and Vpg-Pro genes conferred resistance to PPV (Guo and Garcia, 1997; Ravelonandro et al., 2000; Tavert-Roudet et al., 1998; Wittner et al., 1998) and ToRSV (Sun et al., 2001; Yepes et al., 1996), respectively. A high level of resistance to PPV has been developed in plum (*P. domestica*) and the resistance has been found to persist for over 7 years in orchard conditions (Hily et al., 2004). Importantly, the PDR-based approach makes it possible to engineer resistance to multiple viruses. By expressing the entire CP genes of more than one virus (with each gene being inserted between a constitutive promoter and 3′ termination sequence), transgenic plants resistant to multiple viruses have been generated in a variety of crops, including potato (*Solanum tuberosum* L.) (Kaniewski et al., 1990; Lawson et al., 1990), squash (*Cucurbita pepo* L.) (Fuchs and Gonsalves, 1995; Fuchs et al., 1998), and muskmelon (*Cucumis melo* L.) (Fuchs et al., 1997). Recently, expression of a single chimeric transgene created by fusing the CP gene of turnip mosaic virus (TuMV) to 218- or 110-base pair (bp) of the N gene from tomato spotted wilt virus (ToSWV) was able to confer a resistance to both TuMV and ToSWV in transgenic *N. benthamiana* (Jan et al., 2000). This approach is simple and effective and should be of potential use in the engineering of multivirus resistance in any crop.

The mechanisms of virus resistance through PDR have been found to be either protein-mediated, usually resulting in moderate resistance to a broad range of isolates, or RNA-mediated resulting in extreme resistance limited to a narrow range of closely related isolates (Baulcombe, 1996a). RNA-mediated resistance is homology-dependent and related to posttranscriptional gene silencing (PTGS) (Baulcombe, 1996b). PTGS is marked by high rates of transgene transcription but low steady-state levels of transgene RNA (Baulcombe, 1996a; Dougherty et al., 1994; Lindbo et al., 1993; Prins and Goldbach, 1996). Either viral RNA or transgene transcripts in the host plant serve as both an initiator and target of the silencing mechanism (Baulcombe, 1996b). During the silencing process, the dsRNAs (formed from aberrant transcripts in transgenic plants, RNA transcripts that produce hairpins through inverted repeats, or RNA virus replicate intermediates) are degraded into 21 to 26-nucleotide (nt) small interfering RNAs (siRNA) (Hamilton and Baulcombe, 1999; Hamilton et al., 2002). siRNAs are generated by cleavage of the large dsRNA by a DICER RNase III homologue (Hannon, 2002). siRNA is subsequently incorporated as the specificity determinant into an RNA-induced silencing complex, which guides the degradation of the homologous transgene or viral RNA (Hammond et al., 2000). This produces robust resistance to challenging homologous viruses in a sequence-dependent manner (Hily et al., 2005; Waterhouse et al., 1998).

Demonstration that dsRNA can serve as a potent activator for RNA interference in *Caenorhabditis elegans* Mihi. (Fire et al., 1998) and gene silencing in plants (Chuang and Meyerowitz, 2000; Waterhouse et al., 1998), and that intron-splicing hairpin dsRNA constructs improve the gene silencing efficiency up to 100% (Smith et al., 2000), prompted us to take advantage of this technology for engineering gene silencing-mediated resistance to multiple *Prunus* fruit viruses. Here we used chimeric gene fusions and the intron-splicing hairpin dsRNA strategy to develop a single transgene that would provide resistance against six distinct viruses of *Prunus* fruit. The ability of this construct to induce multivirus resistance was tested in the herbaceous host *N. benthamiana*. The underlying mechanisms of resistance were also characterized.

Materials and Methods

PTRAP6/PTRAP6I AND PLASMID CONSTRUCTION. DNA manipulations were performed according to described protocols (Sambrook and Russell, 2001). To create *PTRAP6*, six primer pairs, based on published sequences of APLPV (AF235033), PDV (AF317089), PMV (AF413919), PNRSV (AF013285), PPV (AJ243957), and ToRSV (U46022), were designed with specific restriction sites incorporated and used for the amplification of ≈400- to 500-bp CP or RdRp gene fragments from pAPLPVCP, pPDVCP, pPNRSVCP, pGA482PPVCP, pGA482ToRSVCP, and PMV70-B1 plasmid DNA. For ease of cloning, the incorporated restriction sites located in the adjacent viral fragments were compatible (e.g., *Bam* HI/*Bgl* II, *Xba*l I/*Nhe* I, and *Sal* I/*Xho* I). The sequences of these primer pair are PPVCP2U (GT GCT AGC GCT CCG CAG TCT TGT TTC CAA ACT), PPVCP2L (CT GGA TCC AGC TTC ACG TGC CCG TAC GGG TGT), ToRSVCPU (CAC GAG CTC GGA TCC GAT ATT GGA CGT CTG TGT GGC CAT), ToRSVCPL (CA TCT AGA ACA AGT TGT AAG TGC TGT CCC TAT), PDVCPU (CT GTC GAC TTT TCC GGT ATG ATA TCT CGT ACC GA), PDVCPL (CA TCT AGA TAA AAC TGA ATC AGG GAC CTC AAC T), PNRSVCPU (GT GCT AGC ACG ACC ACT CTC CCT CAG TTG ATG GGT), PNRSVCPL (CA CTC GAG GTC TTC ATC GAC CAG CAA GAC ATC AGT), APLPVCPU (GT AGA TCT CCT TCT GTG GGT GCC CGT TTA ATT), APLPVCPL (CA GTC GAC GGG CAA CCG AGA CGT TGA AAC CCA), PMVREPU (AC CTCGAG ATG GCT TTC ACC TTC TGC AGA TA), and PMVREPL (CA TCT AGA TGA GCC CTC ATC ATC AGA GCC AT). All information for viral genes, templates, and primer pairs is summarized in Table 1. The amplified fragments were cloned into plasmid pDRIVE (Qiagen, Valencia, Calif.) to create pR2021, pR2022, pR2037, pR2038, pR2039, and pR2041. The sequence identities of cloned gene fragments were confirmed by DNA sequencing. The assembly of *PTRAP6* was accomplished by a multiple step cloning process. Connection between the ToRSV CP fragment isolated from pR2022 and the PNRSV CP fragment isolated from pR2038 generated *PTRAP2A*. Similarly, the connection between the PDV CP and the PPV CP gene fragments and between the APLPV CP and the PMV RdRp gene fragments created *PTRAP2B* and *PTRAP2C*, respectively. The fusion of gene fragments from *PTRAP2A* and *PTRAP2B* created *PTRAP4*, and fusion of *PTRAP2C* and *PTRAP4* fragments created a 2.5-kilobp (kb) *PTRAP6*. As shown in Figure 1, the *PTRAP6* fragment was inserted at *Bam*H I and *Xba* I sites between the CaMV 35S promoter and the *Nos* terminator in pR2059 to create the sense construct *PTRAP6S* and at *Xba* I and *Sac* I sites to create the antisense construct *PTRAP6A*. To create *PTRAP6i,* a second copy of the *PTRAP6* fragment was inserted at *Sac* I and *Xba* I sites next to the first copy of *PTRAP6* in

PTRAP6S in an inverted orientation followed by insertion of an 1.0-kb *Xba* I-*Xba* I DNA fragment containing the first intron of the arabidopsis [*Arabidopsis thaliana* (L.) Heynh.] rubisco activase gene (*RCA*) isolated from plasmid pR825 (Liu et al., 1996) between two copies of *PTRAP6* (Fig. 1). The detailed cloning procedure can be obtained on request.

Plant transformation. The purified plasmids were introduced into *Agrobacterium tumefaciens* (Smith & Towns.) Conn. strain GV3101 through electroporation as described by Mozo and Hooykaas (1991). The transformation of *N. benthamiana* was performed according to the method published by Horsch et al. (1985). Briefly, leaf tissue of 1-month-old *N. benthamiana* seedlings grown in vitro was inoculated with overnight-cultured *A. tumefaciens*. The inoculated leaves were incubated on agar-solidified Murashige and Skoog (MS) medium (Sigma, St. Louis) for 2 to 3 d before being transferred onto selection MS medium supplemented with 1 $mg \cdot L^{-1}$ 6-benzylaminopurine, 0.01 $mg \cdot L^{-1}$ α-naphthaleneacetic acid, 500 $mg \cdot L^{-1}$ carbenicillin, and 200 $mg \cdot L^{-1}$ kanamycin. The culture was maintained in 16/8-h light/dark photoperiod. The regenerated shoots were rooted in the same MS selection medium without plant growth regulators.

Polymerase chain reaction and reverse transcriptase–polymerase chain reaction analysis. Genomic DNA was isolated from greenhouse grown plants using the DNeasy Plant Mini Kit (Qiagen). Approximately 100 ng of genomic DNA was used for polymerase chain reaction (PCR) amplification (30 cycles at 95 °C for 1 min, 62 °C for 1 min, and 72 °C for 1 min) with the primer pairs (ToRSVCPU, PMVREPL) for detection of the *PTRAP6* chimeric gene in transgenic lines.

Total RNA was isolated from plant leaf tissue using the RNeasy Plant Mini Kit (Qiagen) and was treated with DNase to remove contaminated genomic DNA by using the DNA free reagent kit (Ambion, Austin, Texas). RNA quality was examined by gel electrophoresis. The analysis of integrity of *PTRAP6* transcription was performed with the Titan One Tube RT-PCR kit (Qiagen) as described in the manufacturer's directions. One hundred fifty nanograms of total RNA was used for reverse transcriptase–polymerase chain reaction (RT-PCR) amplification with the primer pairs TRSVCPU and PMVREPL (as described previously) that span the entire 2.5-kb *PTRAP6* region. The amplification cycles included 45 °C for 30 min for the reverse transcription step and 95 °C for 15 min for the activation of Hotstart *Taq* DNA polymerase followed by 35 cycles of 94 °C for 1 min, 62 °C for 1 min, and 68 °C for 4 min.

Small interfering RNA analysis. Total RNA was isolated from leaf tissue using TriReagent (Sigma). The ground tissue powder was mixed with 1 mL of Trireagent and the resulting slurries were extracted once with chloroform and precipitated with isopropanol. The dry RNA pellet was dissolved in 50% formamide and ≈20 μg of total RNA was separated in a 0.5X TBE 20% denaturing polyacrylamide gel, electroblotted onto a Hybond-NX membrane (Amersham, Piscataway, N.J.), and crosslinked by ultraviolet (ultraviolet) light Stratalinker (Stratagene, La Jolla, Calif.). Hybridization was performed as described by Hamilton and Baulcombe (1999). Briefly, the membrane was prehybridized with 25 mL of Perfect-Hyb Plus (Sigma) at 42 °C for at least 1 h. Twenty-five nanograms of viral DNA fragments were labeled with ^{32}P isotope using random primers DNA labeling system (Invitrogen, Carlsbad, Calif.), added to the same prehybridization buffer, and incubated at 42 °C for overnight. The hybridized filters were washed twice with 2X SSC, 2% SDS, once with 1X SSC, 1% SDS, and once with 0.5X

Table 1. Viral genes, plasmid templates and primer pairs used for the construction of *PTRAP6*.

Virus	Plasmid template	Viral gene	Primer pair	Reference for template
APLPV	pAPLPVCP	CP	APLPVCPU/APLPVCPL	This work
PDV	pPDVCP	CP	PDVCPU/PDVCPL	This work
PMV	pPMV70-B1	RdRp	PMVREPU/PMVREPL	This work
PNRSV	pPNRSVCP	CP	PNRSVCPU/PNRSVCPL	This work
PPV	pGA482-PPVCP	CP	PPVCP2U/PPVCP2L	Scorza et al., 2001
ToRSV	pGA482-ToRSVCP	CP	ToRSVCPU/ToRSVCPL	Yepes et al., 1996

CP - coat protein, RdRp - RNA-dependent RNA polymerase, APLPV - american plum line pattern virus, PDV - prune dwarf virus, PMV - peach mosaic virus, PNRSV - prunus necrotic ringspot virus, PPV - plum pox virus, ToRSV - tomato ringspot virus.

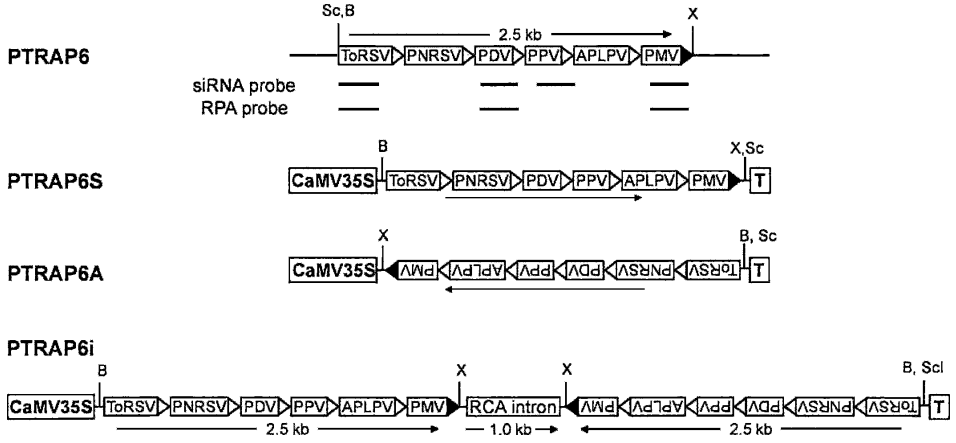

Fig. 1. PTRAP6 constructs. The *PTRAP6* transgene is composed of the CP gene fragments of ToRSV, PNRSV, PDV, PPV, and APLPV and the RdRp gene fragment of PMV. PTRAP6S and PTRAP6A constructs carry one copy of sense- or antisense-oriented *PTRAP6* gene, respectively, under control of CaMV 35S promoter. PTRAP6i is an intron-splicing hairpin dsRNA construct in which two copies of the *PTRAP6* gene were arranged in an inverted repeat under the control of the CaMV 35S promoter. Arrows and numbers above or beneath the depicted constructs indicate the orientations or sizes of gene fragments. The regions used for making DNA or RNA probes are marked under the individual virus gene fragments in the *PTRAP6* gene. The restriction sites depicted are *Sac* I (Sc), *Bam*H I (B), *Xba* I (X); dsRNA = double-stranded RNA; SiRNA = small interfering RNA; kb = kilo bp; CP = coat protein; RPA = ribonuclease protection assay; RdRp = RNA-dependent RNA polymerase; CaMV35S = 35S promoter of cauliflower mosaic virus; T = terminator of nopaline synthase gene; RCA intron = first intron of the arabidopsis rubisco activase (*RCA*) gene; APLPV = american plum line pattern virus; PDV = prune dwarf virus; PMV = peach mosaic virus; PNRSV = prunus necrotic ringspot virus; PPV = plum pox virus; ToRSV = tomato ringspot virus. All constructs are based on binary vector pBIN19.

SSC, 1% SDS at 50 °C for 20 min sequentially. Hybridization signals were detected by STORM 860 (Molecular Dynamics, Sunnyvale, Calif.).

Ribonuclease protection assay. The ribonuclease protection assay (RPA) was performed and detected following the directions provided in the RPA III Kit (Ambion). Briefly, the sense or antisense RNA probe was made by incorporating Biotin-14-CTP (Invitrogen) using the MAXIscript kit (Ambion). The labeled RNA probe was purified in a 0.5X TBE 5% denaturing polyacrylamide gel. The amount of probe used for the protection was determined by an empirical test. The hybridization was carried out with 20 μg of total RNA and an optimal amount of biotin-labeled RNA probe at 42 °C overnight. After RNase digestion, the protected dsRNA was precipitated, denatured, and resolved in a 0.5X TBE 5% denaturing polyacrylamide gel, electroblotted on a BrightStar-Plus positively charged nylon membrane filter (Ambion), and ultraviolet crosslinked. The hybridization signal was detected using the BrightStar BioDetect kit (Ambion) following the provided directions.

Virus inoculation and enzyme-linked immunosorbent assay analysis. ToRSV Yellow bud isolate (ToRSV YB), PDV, and PPV were routinely maintained in *N. benthamiana* host plants. The inoculum was prepared by harvesting symptomatic leaves in 20 mM phosphate buffer (pH 7.6) with 10 mM sodiummdiethyldithio-carbamate trihydrate. R_0, R_1, R_2, and R_3 generation of transgenic plants with ≈10 to 13 cm in height were inoculated with 1:30 to 1:50 diluted inoculum by gently rubbing the upper surface of leaves predusted with carborundum (Fisher Scientific, Pittsburgh). To ensure inoculation efficiency, plants inoculated with PDV were reinoculated 2 weeks later. The PPV inoculation was carried out in a contained greenhouse at the Canadian Food Inspection Agency in Sydney, Canada. Enzyme-linked immunosorbent assay (ELISA) analysis was performed according to the protocols provided in the Durviz PPV, PathoScreen PDV, and PathoScreen ToRSV kits (Agdia, Elkhart, Ind.). One gram of leaf tissue was ground in a mesh bag and tissue sap was diluted 10 times with general extract buffer. One hundred microliters of diluted extracted sample was loaded onto a precoated plate and incubated at room temperature for 2 h. The plate was washed, reacted with enzyme conjugate, and subsequently paraneoplastic pemphigus according to the instructions. The absorbance of ELISA reaction was analyzed in an automated spectrophotometer at 405 nm. For quantitative ELISA analysis of PDV, 100 μg of the inoculated leaf tissues was harvested 5 weeks after first inoculation, ground in 900 μL of general extract buffer in microfuge tubes, and 100 μL of each sample was used for ELISA analysis as directed.

Segregation assay in R_1, R_2, and R_3 seedlings. Progenies from individual transformed plants were screened for segregation of the transgenes as follows: R_1, R_2, and R_3 seeds were surface sterilized in 1.2% sodium hypochlorite for 20 min followed by several washing with sterile distilled water and germinated in agar-solidified MS medium in the presence of 150 $mg \cdot L^{-1}$ kanamycin. Germinating seedlings containing the NPT II gene showed normal development and growth, whereas nontransformed seedlings were stunted and bleached at the cotyledon stage. The kanamycin-resistant/-susceptible ratio was recorded to determine the state of homozygosity.

Results

Construction of chimeric transgene PTRAP6 and analysis of its transcription integrity. Six major *Prunus* stone fruit viruses (APLPV, PDV, PMV, PNRSV, PPV, and ToRSV) were chosen for the targeted engineering of multivirus resistance in this study based on their agricultural importance. To achieve the targeted goal, ≈400- to 500-bp gene fragments (well above the minimum size of 110 bp that was previously reported by Jan et al., 2000) were cloned from the selected regions in CP or RdRp genes of six viruses using PCR amplification and verified by DNA sequencing. The CP and RdRp genes were selected primarily because they have proven to be highly effective for producing virus resistance in plants (Goldbach et al., 2003). The chimeric transgene *PTRAP6* (*P*NRSV, *To*RSV, PDV, PPV, *A*PLPV, and *P*MV) was created by stepwise fusion of five viral *CP* and one *RdRp* gene fragments in the order depicted (Fig. 1). *PTRAP6* did not bear an open reading frame. To make an intron-splicing hairpin construct, two copies of *PTRAP6* were placed in an inverted repeat separated by a spacer intron fragment to create the gene-silencing competent, multivirus targeted carrier *PTRAP6i* (Fig. 1).

Fusion of gene fragments derived from six viruses could unintentionally create false splicing signals or activate cryptic splicing sites in *PTRAP6*, which could be used by plant splicing machinery to remove partial *PTRAP6* transcripts in either strand. As a result, the integrity of *PTRAP6* transcript as well as multivirus resistance would be partially or entirely impaired. To examine this possibility, we made two additional gene constructs, *PTRAP6A* and *PTRAP6S* (Fig. 1); the former expressed the antisense strand, whereas the latter expressed the sense strand of *PTRAP6*. The expression of both constructs in transgenic plants allowed us to address the RNA integrity derived from both strands of the *PTRAP6* tansgene. We introduced *PTRAP6A* and *PTRAP6S* into *N. benthamiana* and characterized the RNA integrity through RT-PCR using a primer pair spanning the entire 2.5-kb *PTRAP6* gene. If splicing or partial splicing occurred within the *PTRAP6* transcript, a smaller size of RT-PCR product or multiple products would be detected. Otherwise, a 2.5-kb unspliced fragment would be expected. Figure 2 showed that neither a smaller size of fragment nor multiple fragments were detected by RT-PCR in the transgenic plants. Instead, an ≈2.5-kb band was detected in sense transgenic line *PTRAP6S*-1 and 3 and the antisense line *PTRAP6A*-1, 2, and 3, indicating that no splicing occurred within *PTRAP6* transcript. The treatment of RNA samples with DNase before RT-PCR amplification and lack of any amplified DNA band from DNase-treated RNA by direct PCR amplification (data not shown) ruled out any possibility that the observed 2.5-kb DNA band amplified by RT-PCR in five of six transgenic plants was attributable to genomic DNA contamination in the RNA samples.

Screening for tomato ringspot virus resistance in R_0 generation. *Nicotiana benthamiana* is highly susceptible to ToRSV (Bitterlin and Gonsalves, 1988) and PPV (Tavert-Roudet et al., 1998; Wittner et al., 1998), and the infected plants develop systemic disease symptoms ideal for the visible screening of resistant phenotypes in transgenic plants. A total of 34 lines of R_0 *PTRAP6i*-transformed plants were generated, and 28 of them were confirmed to carry the *PTRAP6i* transgene by PCR analysis. The confirmed lines were screened

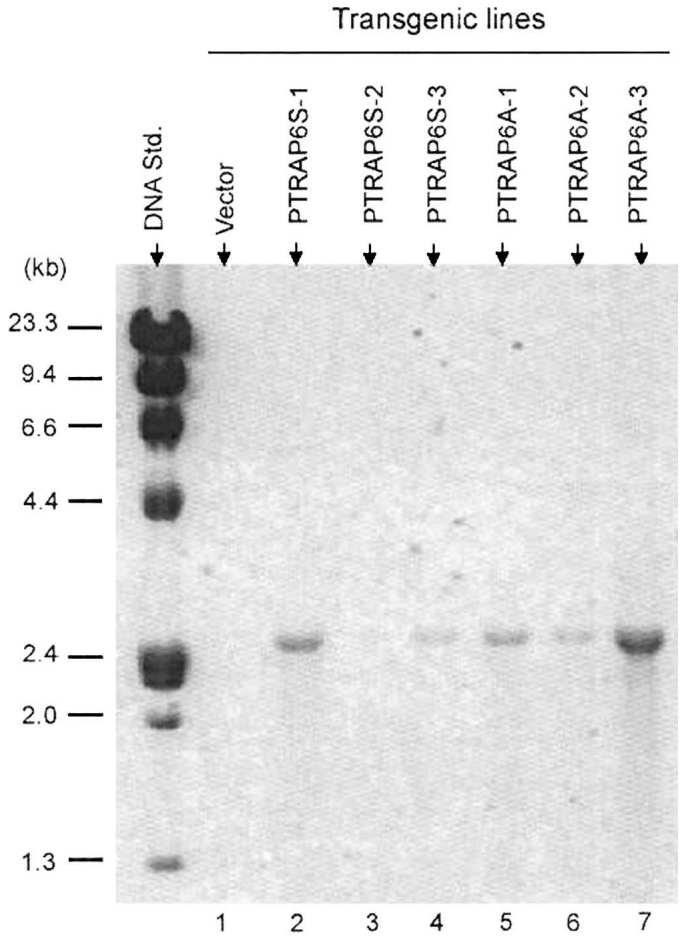

Fig. 2. RT-PCR analysis of *PTRAP6* expression integrity in transgenic *Nicotiana benthamiana* plants. Total RNA was isolated from vector-, *PTRAP6*S-, and *PTRAP6*A-transformed lines and treated with DNase I. Approximately 150 ng of RNA was used for the RT-PCR reaction with *PTRAP6*-specific primer pairs. The color of the gel image is inverted using CorelDraw software (Corel, Dallas, Texas); DNA Std. = λ phage DNA digested by restriction enzymes *Hin*d III, *Bam*H I, and *Xba* I together; kb = kilo bp.

for virus resistance by inoculation with ToRSV YB isolate (98% homology to the transgene). Inoculated control (non-transformed or vector-transformed) plants developed local chlorotic lesions on the inoculated leaves ≈5 d postinoculation (DPI) and severe necrosis in apical shoot tip about 8 DPI, and were completely collapsed 14 DPI. Sixteen of 28 (57%) ToRSV inoculated transgenic lines behaved like susceptible control plants and subsequently died. In contrast, nine of 28 (32%) R_0 lines showed a moderately resistant phenotype with mild visible symptoms and survived long enough to produce seeds. Three of 28 (11%) R_0 lines (designated T24, T25, and T38) developed no visible symptoms or systemic necrosis and were considered highly resistant. These three resistant lines plus one susceptible line T16 were subjected to further analysis. To obtain homozygous progenies, the R_0 lines of T16, T24, T25, and T38 were self-pollinated and their progenies were analyzed for segregation of the NPT II insert. The R_1 progeny analysis showed that the line T16, T24, and T25 carried a single NPT II insert and the line T38 contained at least two inserts (data not shown), which was not used to produce homozygous lines. Seeds from 10 different R_1 plants of line T16, T24, and T25 were germinated in the presence of kanamycin, and at least two R_2 homozygous lines for each transgenic lines (designated T16H1 and 2, T24H1 and 2, and T25H1 and 2) that did not segregate for the transgenic locus were identified and homozygosity of the transgene was confirmed in the R_3 generation. All homozygous lines displayed a normal growth and developmental phenotype and were used in all subsequent studies.

Evaluation of homozygous lines for multivirus resistance. The homozygous lines were further evaluated for resistance to PDV, PPV, and ToRSV infection. At least 10 plants of the R_3 homozygous lines and controls were inoculated with ToRSV YB, PPV-D (96% homology to transgene), and PDV (94% homology to transgene), respectively, and virus symptoms were monitored and analyzed 8 to 40 DPI. Tomato ringspot virus-inoculated control plants developed severe necrosis at the apex within 8 DPI and completely collapsed at 14 DPI, whereas all homozygous plants of T25H1 lines rarely developed visible symptoms (Fig. 3A). Mild local chlorotic lesions in old inoculated leaves occasionally appeared 30 DPI in two of the 10 plants, but newly formed leaves were symptom-free, suggesting that these lines were resistant but not immune to ToRSV. This confirmed the resistant phenotype observed in R_0 plants of the same lines and was also consistent with a typical resistance phenotype (Sun et al., 2001; Yepes et al., 1996). Similarly, the PPV-D-inoculated T25H1 line also displayed a resistant phenotype (Fig. 3B). The inoculated control plants developed chlorotic necrosis in inoculated leaves and stunted growth in younger leaves and the shoot, but the inoculated plants of all homozygous lines showed no chlorotic necrosis and developed normally. The T25H1 line also showed a broad resistance to several PPV isolates, including PPV-C and PPV-M strains (data not shown). The inoculated T24H1 line showed a similar resistance response to both ToRSV and PPV infection (data not shown). PDV was able to infect *N. benthamiana* plants but did not induce any visible symptom (data not shown). Although PDV virus was detected by ELISA in both inoculated controls and homozygous plants, the virus titer detected by ELISA in homozygous lines T24H1 and T25H1 was only 20% to 30% of that detected in control plants (Fig. 3C). These data indicate that these transgenic lines were resistant but not immune to PDV. We were unable to evaluate the resistance of these transgenic lines to three additional viruses (PMV, PNRSV, and APLPV) targeted by *PTRAP6* because they were either unavailable or unable to infect *N. benthamiana*.

Evidence for posttranscriptional gene silencing in highly resistant transgenic lines. PTGS has been established as a host defense mechanisms against viral pathogens (Baulcombe, 2002) and has been widely used for engineering homology-dependent virus resistance in plants (Goldbach et al., 2003). To understand whether PTGS underlies the resistance conferred by *PTRAP6i*, we characterized siRNA production and the abundance of *PTRAP6i* transcripts in both resistant and susceptible lines. *PTRAP6i* was expected to produce an ≈6.0-kb transcript that, owing to its complementary nature, would form a 2.5-kb of *PTRAP6* dsRNA with a 1-kb hairpin loop. The degradation of the dsRNA by Dicer-like RNase should produce siRNAs specific for the six virus gene fragments incorporated in *PTRAP6*. In contrast, the hairpin loop of the intron fragment would not participate in the dsRNA formation and was not expected to be degraded into siRNA species. We first evaluated PPV CP siRNA production in four transgenic lines following a report of PPV CP siRNA in

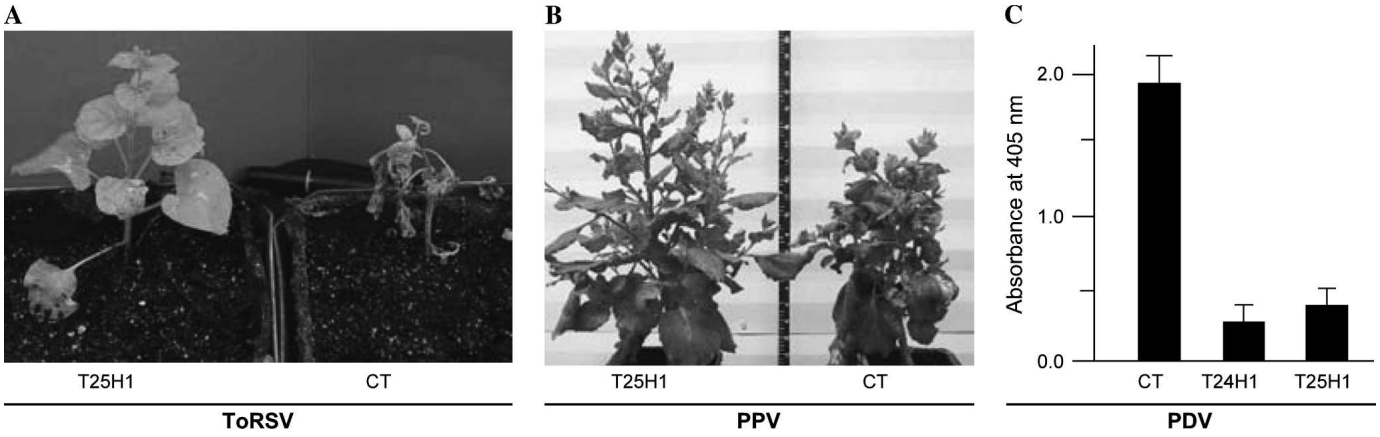

Fig. 3. Resistance of *PTRAP6i* transgenic plants to multiple viruses. Two- to 3-week-old transgenic *Nicotiana benthamiana* plants carrying *PTRAP6i* or vector transgene were challenged with three viruses: (A) tomato ringspot virus (ToRSV), (B) plum pox virus (PPV), and (C) prune dwarf virus (PDV), respectively. The homozygous resistant T24H1 and T25H1 plants were derived from the line T24 and T25, respectively, and susceptible CT plants were derived from the vector-transformed line. Quantitative ELISA analysis for PDV (C) was performed 5 weeks after the first inoculation and the data were obtained from at least four replicates. The photos were taken 40 DPI; CT = control.

resistant transgenic plum (Hily et al., 2005). A PPV CP siRNA species of 21 to 23-nt was specifically detected in RNA samples isolated from leaf tissues of resistant lines T24H1, T25H1, and T38A but not from susceptible line T16H1 (Fig. 4A). Similar results were obtained from R_0 and R_1 generation plants of the same lines (data not shown). Analysis of nine additional moderately resistant lines with the same gene probe showed that PPV CP-specific siRNA was also produced in all lines analyzed (data not shown), indicating that all 12 resistant lines underwent gene silencing. These 12 lines accounted for 43% of the total transgenic lines obtained. Gene-wide siRNA production was subsequently investigated at the 5′, interior, and 3′ areas of the *PTRAP6* gene, corresponding to ToRSV, PDV, and PMV regions (Fig. 1). Figure 4B showed that ToRSV CP-, PDV CP-, and PMV RdRp-specific siRNA species were detected only in the same resistant line T24H1, T25H1, and T38A but not in susceptible line T16H1. As expected, there was no siRNA detected by the probe of the intron (Fig. 4B). Taken together, these data indicate that siRNA production in the resistant lines spans the entire *PTRAP6* transgene and is correlated with virus resistance.

Early studies showed that transgene transcripts in either PPV or ToRSV-resistant transgenic plants were significantly reduced or barely detected (Scorza et al., 2001; Sun et al., 2001; Yepes et al., 1996). To address transgene transcript abundance, we analyzed *PTRAP6i* expression using a RPA with labeled antisense RNA probes of ToRSV CP, PDV CP, and PMV RdRp genes located at the 5′, interior, and 3′ areas of the *PTRAP6* (Fig. 1). Figure 4C showed that the riboprobe of ToRSV CP, PDV CP, and PMV RdRp protected RNA fragments ranging from 410 to 530 nt in size only in susceptible line T16H1 but not in the resistant line T24H1, T25H1, and T38, strongly suggesting that *PTRAP6i* transcripts are undetectable in the resistant lines. To rule out the possibility that failure in protecting RNA fragments in the resistant lines was attributable to either lack of high quality or insufficient amounts of RNA, we performed RPA with the riboprobe of NPT II in the same RNA samples. Because all transgenic lines were selected in the presence of kanamycin, they must contain and actively express the NPT II gene; therefore, the NPT II probe should protect NPT II transcript fragments in both resistant and susceptible lines. Indeed, as shown in Figure 4C, a 350-nt RNA fragment was protected in all four lines tested regardless of virus resistance or susceptibility, indicating that RNA quality and amount used for the RPA assay was of sufficient quality and quantity for the analysis.

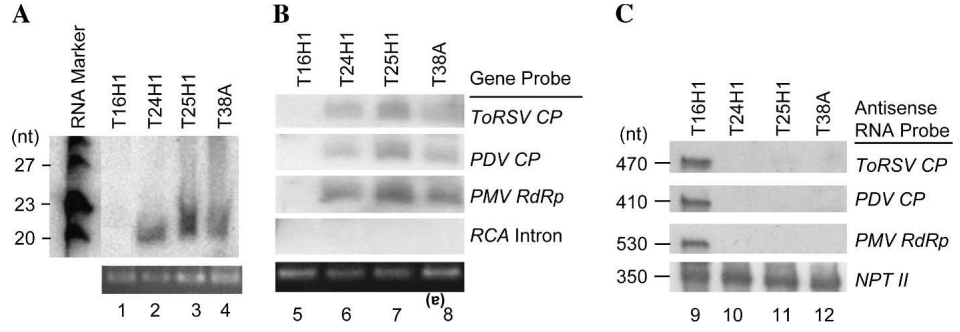

Fig. 4. Analysis of posttranscriptional gene silencing (PTGS) profile in transgenic *Nicotiana benthamiana* plants carrying *PTRAP6i*. (A) RNA gel blot analysis of PPV CP siRNA production in resistant (T24H1, T25H1, and T38A) and susceptible (T16H1) plants. (B). RNA gel blot analysis of transgene-wide siRNA production in transgenic plants using ToRSV CP, PDV CP PMV RdRp, and *RCA* intron gene probes. Ethidium bromide staining of predominant RNA species in the fractioned samples is shown as a loading control in bottom panels of (A) and (B). (C) Ribonuclease protection assay of *PTRAP6i* and NPT II transcript with riboprobes of ToRSV CP, PDV CP, PMV RdRp, and NPT II genes, respectively. T16H1 plants are from a homozygous R_3 generation derived from transgenic line T16. T38A plants were selected as a kanamycin-resistant plant from the R_1 population of transgenic line T38; SiRNA = small interfering RNA; CP = coat protein; RdRP = RNA-dependent RNA polymerase; RCA intron = first intron of the arabidopsis rubisco activase gene; NPT II = neomycin phosphotransferase II; PDV = prune dwarf virus; PMV = peach mosaic virus; ToRSV = tomato ringspot virus; nt = nucleotide.

Discussion

Numerous viral pathogens pose serious threats to stone fruit production. To address this

problem, we developed a gene silencing competent, multivirus targeted carrier, *PTRAP6i*, and characterized its ability to confer multivirus resistance in *N. benthamiana*. Results presented in this study show that *PTRAP6i* was able to confer resistance to all three viruses tested. In initial screenings of R_0 plants, up to 43% showed resistance to ToRSV, exhibiting mild symptom to being symptom-free. This is consistent with previous studies in which phenotypes ranging from a delay of symptoms and reduction in severity to complete immunity were observed in ToRSV CP and VPg-pro transformed populations (Sun et al., 2001; Yepes et al., 1996). Further characterization of two highly resistant lines in the R_3 generation showed they were resistant to PDV, PPV, and ToRSV, three of six viruses targeted by *PTRAP6i* (Fig. 3). As more viruses are available in the future, analyzing their resistance to more targeted viruses as well as different strains of the same virus should provide information about the range, degree, and spectrum of *PTRAP6i*-conferred resistance. Interestingly, we did not observe lines that showed complete immunity. The reason for this has not been investigated. However, the early studies used full-length viral genes for the generation of transgenic plants (Sun et al., 2001; Yepes et al., 1996), but the present study used only 400- to 500-bp viral gene fragments incorporated into *PTRAP6*. The difference of the viral gene length used in the early and present work could explain the lack of immune phenotypes observed in this study.

Detailed analyses revealed that the resistance conferred by *PTRAP6i* is based on PTGS. The early studies showed that high levels of resistance were inversely related to steady-state levels of transgene transcript in resistant lines harboring PPV CP, ToRSV CP, or VPg-pro transgenes, suggesting that PTGS functions as a resistance mechanism (Scorza et al., 2001; Sun et al., 2001; Yepes et al., 1996). The direct evidence to support PTGS-mediated resistance against stone fruit viruses was from the characterization of PPV-resistant transgenic plum in which transgene-specific siRNA was detected and correlated to virus resistance (Hily et al., 2005). The present study presents several lines of evidence to support the occurrence of PTGS in resistant *N. benthamiana* lines. First, transgene-wide siRNA production (ToRSV CP, PPV CP, PDV CP, and PMV RdRp) was detected only in resistant but not in susceptible lines (Fig. 4A, B). Second, no intron-specific siRNA was detected in the same resistant lines, although the intron was part of *PTRAP6i* transgene (Fig. 4B). Third, the transgene transcript was detectable only in susceptible lines but not in resistant lines analyzed (Fig. 4C). Based on these findings, it seems clear that PTGS is the cause of multivirus resistance in our system. Consistent with resistant phenotypes, siRNA production was also detected in nine moderately resistant lines (data not shown). Our study that achieved ≈43% (12 of 28) of gene silencing/resistance frequency through *PTRAP6i* supports an early finding that the intron-splicing hairpin dsRNA construct was more efficient than either sense or antisense constructs in inducing gene silencing/virus resistance (Smith et al., 2000).

The key to achieving multivirus resistance through gene silencing is to introduce multiple viral gene fragments that simultaneously elicit PTGS. Although coexpression of gene fragments from distinct viruses can confer multivirus resistance (Fuchs and Gonsalves, 1995; Fuchs et al., 1998; Lawson et al., 1990), the process of cloning multiple transcriptional units into a single vector is tedious and requires multiple promoters and transcriptional terminator elements, which tend to present technical difficulty for cloning and cause structural instability attributable to rearrangement. Fusing several gene fragments as a single chimeric transgene obviates the problem associated with a coexpression approach and achieves at least equal efficiency of virus resistance (Jan et al., 2000). *PTRAP6i* developed through the combination of the single chimeric transgene and intron-splicing hairpin dsRNA strategies offers several advantages over existing approaches. It targets up to six viruses with high gene silencing efficiency and dsRNA formation and degradation; therefore, *PTRAP6i*-mediated resistance would not likely interfere with physiological and biochemical processes or pathways in plants, and it would mitigate concerns related to issues of RNA-mediated virus recombination. Importantly, the high efficiency of gene silencing/virus resistance conferred by *PTRAP6i* will expedite the development of multivirus-resistant stone fruit cultivars. Hence, *PTRAP6i* can serve a powerful means for engineering multivirus resistance in stone fruit species without the need to produce a large number of transgenic lines.

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