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Genetic Resources

Analysis of RNA-Mediated Gene Silencing Using a New Vector (pKNOCKOUT) and an In Planta *Agrobacterium* Transient Expression System*

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Abstract. A hairpin RNA (hpRNA) vector, pKNOCKOUT (pKO) has been constructed to facilitate the analysis of posttranscriptional gene silencing (PTGS) in an *Agrobacterium*-mediated transient expression system developed for tobacco. The pKO binary vector was tested by cloning a firefly luciferase (*Photinus pyralis*) gene segment in sense (sFLUC), antisense (aFLUC), and inverted repeat (ihpFLUC) orientations. The inverted repeats of the target gene are separated by the castor bean catalase intron (CbCi) and, when transcribed and spliced, produce a self-complementary hpRNA. hpRNA-mediated gene silencing exploits a cellular mechanism that recognizes double-stranded RNA (dsRNA) and subjects it, and homologous mRNA molecules, to sequence-specific degradation. Agrobacteria harboring compatible plasmids, pE1778-RiLUC (*Renilla luciferase* normalization construct) and pTCaMV35S-FiLUC (functional firefly luciferase test construct), were co-infused with different variants of pKO-FLUC plasmids into *Nicotiana benthamiana* and *Nicotiana tobaccum* leaf tissues. Reduced firefly luciferase reporter gene activity (from pTCaMV35S-FiLUC) indicated gene silencing and was observed in leaf tissues co-infused with pKO-sFLUC (50% reduction), pKO-aFLUC (85% reduction), or pKO-ihpFLUC (96% reduction) agrobacteria lines. Gene silencing was observed at different times postinfusion in leaves from both *Nicotiana* species. The hpRNA-mediated interference with FiLUC reporter gene expression, as measured by reduced firefly luciferase activity, was found to also suppress silencing of the cotransferred *Renilla reniformis* luciferase normalization reporter gene, resulting in reproducibly elevated RiLUC activity. This suppression effect was reduced by lowering the percentage of infused pKO-ihpFLUC agrobacteria without markedly effecting hpRNA-mediated gene silencing of FiLUC (all pKO-ihpFLUC dilutions produced >90% reduction of FiLUC activity). Viral suppressors of PTGS, such as p19 and HcPro, were found to reduce the RNAi effect of hpRNA-mediated gene silencing. To our knowledge, this is the first demonstration that excess amounts of targeted dsRNA can result in nonspecific suppression of PTGS of an unrelated, co-infused reporter gene in plants.

Key words: antisense, *Nicotiana, Photinus pyralis,* posttranscriptional gene silencing, *Renilla reniformis,* reporter gene, RNA interference

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Abbreviations: CAM, coelenterazine assay media (sea pansy); CaMV35S, cauliflower mosaic virus 35S transcript; CbCi, castor bean catalase intron; dsRNA, double-stranded RNA; dpi, days postinoculation; DTT, dithiothreitol; GUS, β-glucuronidase; hpRNA, hairpin RNA; ihpRNA, intron hairpin RNA; Km, kanamycin; LB, Luria-Bertani; FLUC, *Photinus pyralis* (firefly) luciferase gene segment; FiLUC, *Photinus pyralis* (firefly) intron-modified luciferase; LAM, luciferin assay media (firefly); miRNA, microRNA; Nos^t, nopaline synthase terminator; pBS, pBluescript II (SK+); PTGS, posttranscriptional gene silencing; RiLUC, *Renilla reniformis* (sea pansy) intron-modified luciferase; RLUC, *Renilla reniformis* (sea pansy) luciferase; RNAi, RNA interference; RLU, relative light unit; siRNA, small interfering RNA; T-DNA, transferred DNA

Introduction

RNA silencing is a collective term that involves a series of cellular events leading to a reduction of gene expression through a sequence-specific RNA-mediated process. RNA silencing occurs in many organisms and is manifested in one form as posttranscriptional gene silencing (PTGS) (predominantly used to describe plant- and plant-virus–related silencing events) or in animals as RNA interference (RNAi) (reviewed by Baulcombe, 1996; Depicker et al., 1997; Pickford et al., 2003; Susi et al., 2004). In plants, RNA silencing is known to control the developmental regulation of plant gene expression through the production of microRNA (miRNA) (Bartel et al., 2003) and serves as a defense mechanism against invading viruses (Voinnet, 2001).

Hairpin RNA (hpRNA)–mediated gene silencing exploits the cellular mechanisms that recognize double-stranded RNA (dsRNA) and subject homologous mRNA to sequence-specific degradation. Recent work has demonstrated the potential for hpRNA constructs that produce dsRNA to efficiently silence targeted gene expression (Akashi et al., 2004; Scattat et al., 2004; Wesley et al., 2003). The DNA sequences that encode the self-complementary regions of hpRNA constructs form an inverted repeat (Waterhouse et al., 1998), which, after transcription, forms dsRNA that is processed by DICER-like proteins (Xie et al., 2003), forming small RNAs that facilitate targeted PTGS. Intron-containing constructs (e.g., intron hairpin RNA [ihpRNA]) are most effective at inducing gene silencing and can produce independent transgenic lines of which 90-100% exhibit silencing (Wesley et al., 2001). Sense and antisense RNAs are also able to promote PTGS (Van Houdt et al., 2000); however, these forms of RNA usually result in a much smaller proportion of silenced lines (15-30%) (Smith et al., 2000; Wesley et al., 2001). Despite many studies of transgene-induced silencing in stable transformants, very little is known about how sense, antisense, and hpRNA constructs work in transient expression systems.

Agrobacterium-mediated transient gene expression has emerged as a valuable tool to study PTGS phenomena in plants (Johansen et al., 2001; Van der Hoorn et al., 2000). PTGS is an important factor in any transient gene expression system and significantly reduces the efficiency of *Agrobacterium*-mediated transient gene expression (Voinnet et al., 2003). This limitation can be at least partially overcome by using virus-encoded suppressor proteins, such as p19 and HcPro, which have been shown to reduce the effects of PTGS, enhancing reporter gene expression levels several fold (Brigneti et al., 1998; Voinnet et al., 1999).

The intron-containing firefly luciferase (FiLUC; *Photinus pyralis*; Mankin et al., 1997) and sea pansy (RiLUC; *Renilla reniformis*; Cazzonelli et al., 2003) reporter genes have proven to be useful tools for the study of PTGS in transient expression systems (Akashi et al., 2001; Reynolds et al., 2004).

Here we report the construction of a new silencing binary vector, pKNOCKOUT, to facilitate a detailed analysis of PTGS as it impacts the firefly and sea pansy luciferase reporter genes in an *Agrobacterium*-mediated transient expression system. The pKO vector has been used to deliver sense, antisense, and hpRNA targeting FiLUC activity within leaf tissues of 2 *Nicotiana* species transiently expressing firefly luciferase. The impact of these inactive firefly luciferase transcripts on expression of both the targeted (firefly luciferase) and a non-homologous *Renilla* luciferase were examined.

Materials and Methods

Plasmid construction and structure

Plasmid constructs (Figure 1) were prepared by using standard cloning techniques (Sambrook et al., 2001). Test (pTCaMV35S-F, Figure 1B) and silencing (pKO-based, Figure 1C-F) binary plasmids are based on the pPZP200 binary vector (Hajdukiewicz et al., 1994). A marker gene for selection of transgenic plants is positioned near the T-DNA left border in all constructs. In the test- and pKO-derived plasmids, the selectable marker consists of the *bar* gene, encoding phosphinothricin acetyl transferase (GenBank accession number, AX235900), controlled by a peanut chlorotic streak virus promoter (–240 to +1 bp; Maiti et al., 1998) and a cauliflower mosaic virus 35S (CaMV35S) transcription termination signal. The pE1778-based plasmid pE1778-SUPER-R (Figure 1A) uses kanamycin (Km) resistance provided by a nopaline synthase promoter–driven neomycin phosphotransferase II gene with the T-DNA gene 7 terminator (Becker et al., 1992; S. Gelvin, personal communication).

The pTCaMV35S-F (CF, Figure 1B) binary plasmid contains an intron-modified firefly luciferase gene (Mankin et al., 1997) controlled by a modified CaMV35S promoter (–299 to –99 joined to –46 to +1) (Benfey et al., 1990) and the nopaline synthase terminator.

The pTCaMV35S-F plasmid (Figure 1B) served as the starting point for production of the pKNOCKOUT (pKO) silencing vector. The castor bean catalase intron (CbCi) used in GUSi (Ohta et al., 1990) was amplified by using PCR with synthetic primers that incorporate 4 unique restriction sites at either side of consensus eukaryotic splicing sites. The FiLUC gene was removed by a *Bam*HI and *Xba*I digest and replaced with the CbCi. The resulting vector (pKO) was sequenced to confirm that the eukaryotic splicing sites recognized by the plant spliceosome (Figure 1C) were retained during the cloning process. A suite of pKO silencing vectors was constructed to study PTGS of the FiLUC reporter gene. Antisense or sense orientations of a segment (+435 to +1631 bp) of the luciferase reporter gene (Mankin et al., 1997) were cloned on either side of the CbCi to generate pKO-aFLUC (Figure 1D) and pKO-sFLUC (Figure 1E). Antisense and sense orientations of the luciferase segment were combined in a single

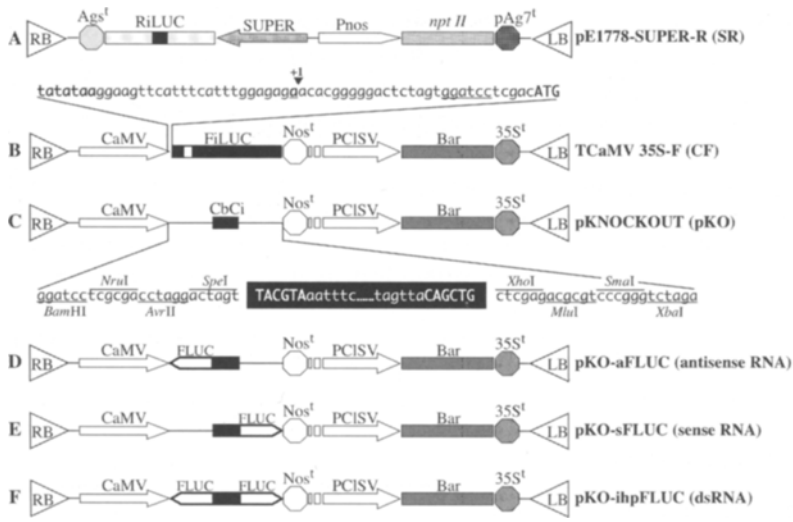

Figure 1. Schematic diagram showing the construction of normalization, test, and silencing binary vectors. (A) pE1778-RiLUC (SR): normalization plasmid. (B) pTCaMV35S-F (CF): Test construct showing the CaMV35S promoter and FiLUC gene junction. The TATA box (bold), ATG (upper case/bold), and start of transcription (underlined) are depicted. (C) pKNOCKOUT (pKO): Silencing vector showing the sequence at the multiple cloning site (restriction sites underlined) and the exon-intron junctions at the CbCi intron (white font, consensus splicing recognition sequences are in uppercase). (D) pKO-aFLUC: Antisense silencing construct showing the FLUC gene inserted into the 5′ side of the CbCi in the antisense orientation. (E) pKO-sFLUC: Sense silencing construct showing the FLUC gene inserted into the 3′ side of the CbCi in the sense orientation. (F) pKO-ihpFLUC: Hairpin silencing construct showing the FLUC gene inserted into both the 5′ and 3′ ends of the CbCi, creating an inverted repeat with sense and antisense orientations of the FLUC gene segment. RB, T-DNA right border; LB, T-DNA left border; Ags^t, agropine synthase terminator; RiLUC, intron-containing *Renilla* luciferase coding region; SUPER, super promoter; Pnos, nopaline synthase promoter; nptII, neomycin phosphotransferase II coding region; pAg7^t, T-DNA *g7* terminator; CaMV, CaMV35S promoter; IV2, potato *ST-LS1* intron; FiLUC, intron-containing firefly luciferase coding region; Nos^t, nopaline synthase terminator; PClSV, peanut chlorotic streak virus promoter; Bar, phosphinothricin acetyl transferase coding region; 35S^t, CaMV35S terminator; CbCi, castor bean catalase intron; FLUC, firefly luciferase coding region (partial).

plasmid (luciferase sequence present on both sides of the CbCi to create an inverted repeat), producing the vector pKO-ihpFLUC (Figure 1F).

The normalization vector, pE1778-SUPER-RiLUC (SR, Figure 1A), was prepared as described by Cazzonelli and Velten (2003). SR harbors a CbCi-modified *Renilla* luciferase reporter gene controlled by the synthetic super promoter (Ni et al., 1995) within the binary vector pE1778 (Becker et al., 1992; S. Gelvin, personal communication). The pIG121 plasmid, a CaMV35S::β-glucuronidase (GUS)–intron binary vector, does not directly impact FiLUC silencing through production of a transcript homologous to the luciferase target and was used as a negative (nonsilencing) control. pIG121 is based on the pBI101 binary vector, and its detailed structure is described elsewhere (Akama et al., 1992). The plasmids containing viral suppressors of gene silencing (pBIN61-HcPro and pBIN61-p19) are described by Voinnet et al., 2003. Predicted DNA sequence files for all the plasmids used in this research are available from the authors upon request.

Luciferase detection in tobacco leaf tissues infused with agrobacteria

Binary vectors were electroporated into EHA105 electrocompetent agrobacteria as described by Walkerpeach and Velten (1994). The pE1778-RiLUC (SR) line of agrobacteria was subsequently cotransformed with the compatible pTCaMV35S-F (CF) plasmid, producing the CF/SR line. Agrobacteria containing CF/SR and separate lines harboring one of the silencing plasmids (pKO-sFLUC, pKO-aFLUC, or pKO-ihpFLUC) or negative control plasmid (pIG121: GUS-intron) were grown as individual cultures at 28°C in Luria-Bertani (LB) media containing the appropriate antibiotic selection (25 μg/mL Km sulfate or 100 μg/mL spectinomycin) until each culture reached an OD_{600} of 0.8. The resulting cultures were independently centrifuged at 8000 *g* for 15 min, washed, and resuspended in an equal volume of infiltration media (50 mM MES [pH 5.6], 0.5% glucose, 2 mM $NaPO_4$, 100 μM acetosyringone). *Nicotiana tobaccum* and *Nicotiana benthamiana* plants were grown in hydroponics solution under artificial lighting with a 16/8 h photoperiod in an environmentally controlled growth room (22°C). Multiple individual leaves (2 leaves per construct) were mechanically infused with agrobacteria by pressing the tip of the syringe against the lower surface of the leaf and applying gentle pressure to the tip of the plunger. Quantitative measurement of luciferase activity was achieved 2-5 d postinoculation (dpi) by using an in vivo floating leaf-disk assay (Cazzonelli and Velten, manuscript in preparation). For each assay, 4 leaf disks (4 mm diameter) per infusion were floated on luciferase assay media (luciferin assay media [LAM] for FiLUC and coelenterazine assay media [CAM] for RiLUC) in separate wells of a 96-well white-walled microtiter plate, and light production from each disk was measured over time with a FLUOstar Optima luminometer from BMG Lab Technologies Inc. LAM buffer contains 1 mM luciferin (diluted from a 100-mM stock dissolved in 20 mM MES [pH 5.6]), 50 mM MES (pH 5.6), 0.5% glucose, 2 mM $NaPO_4$, 0.5% v/v DMSO. CAM buffer contains 11.8 μM coelenterazine (diluted from a 2.36-mM stock dissolved in methanol), 25 mM glucose, 0.1 M $NaPO_4$ (pH 8), 0.05 M NaCl, 1 mM EDTA, 10 mM dithiothreitol (DTT). The maximum peak of light emission over a 90-min period was determined and referred to as the relative light units (RLUs).

Results and Discussion

Design of the pKO silencing vectors

pKO was designed as a generic silencing vector intended to produce nonfunctional or "aberrant" RNA transcripts to facilitate the analysis of PTGS during *Agrobacterium*-mediated transient expression and in stably transformed plants. pKO is based on the efficient and stable pPZP200 binary vector that has a proven utility in plant transformation (Hajdukiewicz et al., 1994). Oriented downstream from the T-DNA right border are a CaMV35S promoter, a multiple cloning site, and a 3′ nopaline synthase terminator (Figure 1C). The multiple cloning site is bisected by the CbCi, which provides eukaryotic splicing sequences that are recognized by the plant spliceosomal machinery, enabling efficient post-transcriptional removal of the intron (Mankin et al., 1997; Vancanneyt et al., 1990). The CbCi was chosen on the basis of its successful use in an intron-

containing version of the sea pansy luciferase gene (RiLUC). The CbCi-containing RiLUC gene was demonstrated to be nonfunctional in agrobacteria and active in planta (Cazzonelli et al., 2003). The same consensus exon-intron border sequences used for RiLUC were incorporated into the construction of pKO.

The multiple cloning site in pKO facilitates insertion of target gene fragments in both the antisense (Figure 1D) and sense (Figure 1E) orientations, or a combination of sense and antisense orientations can be combined to create a hairpin inverted repeat (Figure 1F). Unique restriction endonuclease sites 5′ (*Bam*HI, *Nru*I, *Avr*II, *Spe*I) and 3′ (*Xho*I, *Mlu*I, *Sma*I, *Xba*I) of the CbCi provide multiple options during the cloning of targeted gene segments. Gene segments can be PCR amplified by using primers that contain these unique restriction sites and cloned directly into pKO. An added advantage of pKO is that it contains a plant selectable marker gene (*bar* herbicide resistance). Inter-plasmid sequence duplication was avoided during the design of pKO and CF plasmids. Unique terminators, marker/reporter genes, and promoters were used during vector construction to avoid bacterial homologous recombination, which can occur when repeated sequences are included in the same or compatible plasmids. Both the test (CF, Figure 1B) and pKO (Figure 1C-F) origins of replications are compatible with the normalization plasmid (SR, Figure 1A), allowing their propagation in a single agrobacteria line. This facilitates the combination of agrobacteria lines harboring CF/SR and silencing (pKO-based) gene constructs prior to infiltrating tobacco leaf tissues.

Sense-, antisense-, and ihpRNA-mediated impact on PTGS during transient expression of firefly luciferase

Transient expression within *N. tobaccum* and *N. benthamiana* leaves infused with agrobacteria allows rapid and quantitative measurement of transgene activity, including the affect of plant gene silencing systems on transgene expression. By using compatible binary vectors and mixed agrobacteria cultures, multiple transgenes can be simultaneously expressed, providing a simple and accurate mechanism for exploring in planta gene silencing. Foreign genes expressed during agrobacteria infusion are clearly subject to significant levels of PTGS (Johansen et al., 2001; Van der Hoorn et al., 2000; Figure 3A). By using the pKO sense, antisense, and hairpin luciferase constructs, it is possible to examine the impact of the presence of various nonfunctional forms of luciferase mRNA on FiLUC activity within infused tissues.

Simultaneous production of both the functional (active luciferase product) and inactive (no active luciferase produced) firefly luciferase mRNAs was accomplished by infusing an agrobacteria mixture in which 50% of the bacteria contained compatible firefly and *Renilla* luciferase plasmids (CF/SR, the only source of active luciferase enzymes) and 50% harbored 1 of 3 silencing plasmids (pKO-sFLUC, producing an inactive sense transcript; pKO-aFLUC, producing an inactive antisense transcript; or pKO-ihpFLUC, producing an inactive intron-hairpin transcript) or a negative control plasmid (pIG121, producing a non–firefly-silencing GUS mRNA). Firefly and sea pansy luciferase activities were determined 66 and 138 h after agrobacteria-infiltration by using a floating leaf disc assay (Cazzonelli and Velten, manuscript in preparation). The average RLUs of

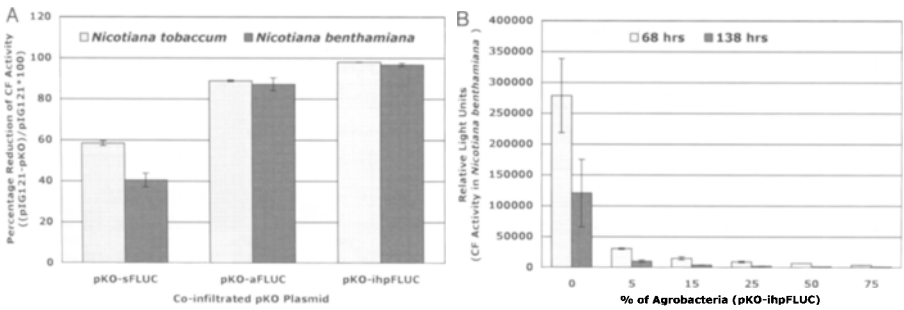

Figure 2. PTGS of the FiLUC reporter gene using pKO silencing vectors in *Nicotiana tobaccum* and *Nicotiana benthamiana*. **(A)** The percentage reduction in FiLUC activity levels using sense-, antisense-, and hairpin-encoding RNA triggers of PTGS. Tobacco leaves from independent plants (n=2) were infused with an agrobacteria-mixture containing 50% of CF/SR and 50% of one of the following: pIG121, sense (pKO-sFLUC), antisense (pKO-aFLUC), or hairpin (pKO-ihpFLUC) binary vectors. The average RLU from independent leaf discs assays (n=4) was determined 66 and 138 h after agroinfiltration. The RLUs are expressed as a percentage reduction of CF/SR+pKO-ihpFLUC activity relative to CF/SR+pIG121 ($[RLU_{pIG121} - RLU_{pKO}]/RLU_{pIG121} \times 100$). pIG121 serves as a control binary vector. Error bars represent the standard error determined from the average of 66- and 138-h measurements. **(B)** Effects of increasing the percentage of an agrobacteria line harboring pKO-ihpFLUC in *N. benthamiana*. Tobacco leaves from independent plants (n=2) were infused with an agrobacteria line containing 25% CF/SR and 5%, 15%, 25%, 50%, and 75% of pKO-ihpFLUC. Agrobacteria harboring pIG121 were used to make the total amount equal to 100%. RLUs were measured 66 and 138 h after agroinfusion. Error bars represent the standard error from individual leaf disc assays (n=2) from 2 separate leaf infusions (n=2).

the 66- and 138-h measurements are presented as the percentage reduction of CF (FiLUC) activity in the presence of the FLUC silencing constructs (pKO-based) relative to the nonsilencing pIG121 control ($[RLU_{pIG121} - RLU_{pKO}]/RLU_{pIG121} \times 100$), Figure 2A).

All 3 pKO-based constructs (producing nonfunctional FLUC transcripts) were able to markedly increase the degree of silencing experienced by the functional firefly gene in both plant species, with the sense, antisense, and hairpin constructs reducing CF/SR+pIG121 control activity by 40-58%, 87-89%, and 96-98%, respectively. Akashi and colleagues had previously reported using plasmid vectors containing a 300-bp FLUC gene segment in the sense, antisense, and inverted repeat orientations to silence FLUC activity within electroporated BY2 tobacco suspension cells (Akashi et al., 2001). Their sense- and antisense-encoding constructs reduced expression by 25-40% in comparison to the inverted repeat construct, which lowered FLUC activity by 85%. Although the BY2 results are similar to those obtained by using agrobacteria infusion and the pKO vector system, all 3 versions of the pKO silencing vectors produced a higher degree of silencing than that observed in the cultured cells with the corresponding sense, antisense, and hairpin constructs.

The reasons for the difference between our findings and those of Akashi et al. (2001) are not immediately evident. It is possible that construct design (the length of the FLUC gene [~1.5 kb] used here is much greater than the 300- and 500-bp segments used by Akashi and colleagues) is a contributing factor. However, Akashi and colleagues report saturation of dsRNA silencing in their transient expression system despite having only used a 300-bp FLUC segment. The

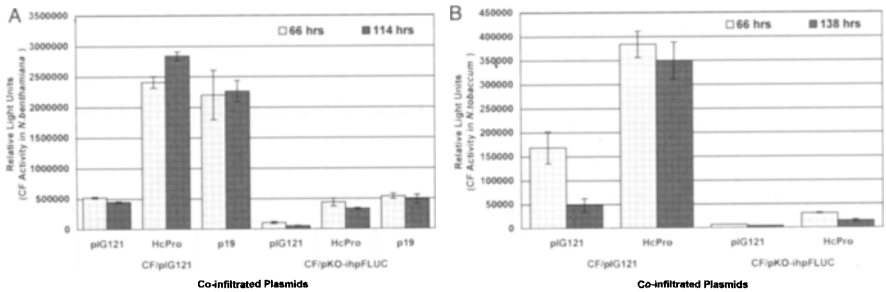

Figure 3. The effects of p19 and HcPro viral suppressors of PTGS on transient FiLUC activity levels in *Nicotiana benthamiana* and *Nicotiana tobaccum*. HcPro (pBIN19-based binary vector encoding the helper component protein [HcPro] from potato virus Y [PVY], driven by the CaMV35S promoter) (Brigneti et al., 1998). p19 (pBIN19-based binary vector encoding the p19 protein from tomato bushy stunt virus [TBSV], driven by the CaMV35S promoter) (Voinnet et al., 2003). **(A)** The effects of HcPro and p19 on CF/SR+pIG121 and CF/SR+pKO-ihpFLUC–mediated gene silencing in *Nicotiana benthamiana*. Tobacco leaves from independent plants (n=2) were infused with an agrobacteria mixture containing 50% CF/SR, 25% pIG121 or pKO-ihpFLUC, and 25% p19 or HcPro. The average RLU from leaf disc assays (n=4) was determined 66 and 138 h after agroinfiltration. Error bars represent the standard error of separate leaf infusions (n=2). **(B)** The effects of HcPro on CF/SR+pIG121 and CF/SR+pKO-ihpFLUC–mediated gene silencing in *Nicotiana tobaccum.* Tobacco leaves from independent plants (n=2) were infused with an agrobacteria mixture containing 50% CF/SR, 25% pIG121 or pKO-ihpFLUC, and 25% HcPro. The average RLU from leaf disc assays (n=4) was determined 66 and 90 h after agroinfiltration. Error bars represent the standard error of separate leaf infusions (n=2).

difference may also be attributed to the phenomenon that segments of a target mRNA display varying degrees of sensitivity to RNA-based silencing. A comprehensive analysis of RNAi was undertaken in transfected cultured mammalian cells by using 180 synthetic short-interfering RNAs (siRNA) that span a FLUC gene segment (+1434 to +1631bp; accession # U47298) (Reynolds et al., 2004). Many effective targets sites were determined, with 78% of the siRNAs inducing more than 50% silencing and 24% of the siRNA targets inducing over 95% silencing. An equally likely explanation is that the method of transgene delivery (agrobacteria infusion versus BY2 cell culture systems) impacts the effectiveness of the silencing constructs.

The effects of increasing the proportion of an agrobacteria mixture containing pKO-ihpFLUC were evaluated in *N. benthamiana*. Leaves from duplicate independent plants were infused with a mixed culture containing 25% CF/SR and 5%, 15%, 25%, 50%, and 75% of pKO-ihpFLUC (the remainder in each case being the nonsilencing pIG121 line). Firefly and *Renilla* luciferase activities were determined 68 and 138 h after infusion (Figure 2B). A very sharp decrease in CF activity was observed by using as little as 5% pKO-ihpFLUC within the infused culture. Increasing the percentage (15-75%) of agrobacteria harboring pKO-ihpFLUC resulted in continued, albeit smaller, reductions in CF activity levels relative to the 5% culture. It is clear that pKO-ihpFLUC effectively silences FiLUC activity at very low agrobacteria densities, confirming hairpin-encoding constructs as very potent activators of PTGS in transient expression systems.

The pKO-ihpFLUC construct is capable of reducing FiLUC activity (96-98% silencing) in our transient assay system with levels comparable to those observed by using hairpin-encoding constructs transformed into stable transgenic

plants (90-100% silencing; Scattat et al., 2004; Wesley et al., 2001). Sense and antisense constructs reduced FiLUC activity (40-89%) in the transient assay system to a much greater extent then that observed in stable transformed individuals (15-30% silencing; Wesley et al., 2001), possibly because of overall higher levels of transgene expression within the infused leaf tissues (Cazzonelli, unpublished results). Analysis of our sense-, antisense-, and hairpin-encoding FLUC constructs in stable transformed tobacco expressing a functional firefly luciferase protein will help determine the reason for these differences.

Viral suppressors reduce PTGS of the FiLUC gene

To confirm that the observed reduction in luciferase activity results from gene silencing, the effects of p19 and HcPro (Voinnet et al., 2003), viral suppressors of PTGS, on transient FiLUC activity were determined. Agrobacteria mixtures containing CF/SR and the nonsilencing pIG121 or pKO-ihpFLUC constructs were infused into *N. benthamiana* and *N. tobaccum* leaves. Agrobacteria lines contained 50% CF/SR, 25% pIG121 or pKO-ihpFLUC, and 25% pBIN61-p19 or pBIN61-HcPro (Figure 3). The average RLU from independent leaf discs was determined 66, 114, and 138 h after infiltration. Both p19 and HcPro effectively suppressed PTGS of CF/SR+pIG121 and CF/SR+pKO-ihpFLUC, raising FiLUC activities several fold (4- to 7-fold in *N. benthamiana* [Figure 3A] and 3- to 4-fold in *N. tobaccum* [Figure 3B]). Although the enhancement of FiLUC activity (~3- to 7-fold) is similar in CF/SR+pIG121 and CF/SR+pKO-ihpFLUC agrobacteria lines, the absolute RLUs of the CF/SR+pKO-ihpFLUC infusions remain considerably less than those obtained from the CF/SR+pIG121 infusions.

The lower nonsuppressed (no suppressor) values in CF/SR+pKO-ihpFLUC relative to CF/SR+pIG121 are consistent with overall enhanced silencing in the presence of the hairpin construct (Figure 3A). Why the viral suppressors do not more effectively alleviate the strong silencing induced by the hairpin-encoding pKO construct is not immediately clear. The hairpin-encoded dsRNA produced by CF/SR+pKO-ihpFLUC may possibly stimulate the PTGS cellular mechanism to the degree that p19 and HcPro are unable to overcome this effect. However, it is interesting to note that in *N. benthamiana* both HcPro and p19 were able to suppress CF/SR+pKO-ihpFLUC–induced PTGS to the extent that RLU values are returned to levels comparable to those seen with just the CF/SR+pIG121 (no suppressor) agrobacteria line (Figure 3A). This effect was not observed with HcPro in *N. tobaccum* (Figure 3B) (the p19 plasmid cannot be used with *N. tobaccum* because of rapid death and necrosis that occurs within the infused leaf tissue).

Our data (Figure 3A-B) clearly indicates that viral suppressors of PTGS alleviate CF/SR+pKO-ihpFLUC– and CF/SR+pIG121–induced silencing in the transient expression system in both *Nicotiana* species. Further analysis is required to understand the molecular basis behind the limited suppression observed with the CF/SR+pKO-ihpFLUC infusions. Testing of viral suppressors on sense- and antisense-induced PTGS of FiLUC activity may help provide a better understanding of this effect.

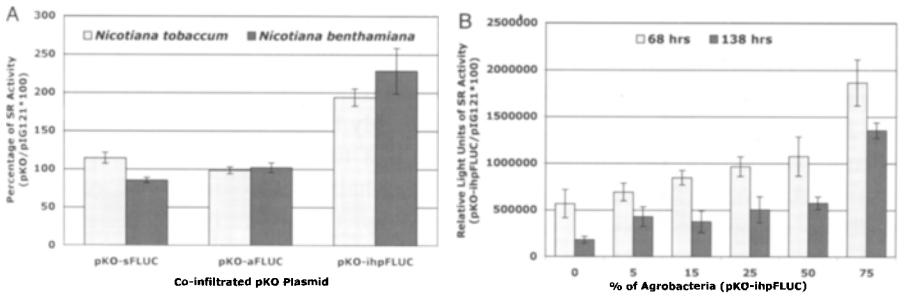

Figure 4. Nonspecific suppression of PTGS of the RiLUC normalization reporter gene. **(A)** The percentage of RiLUC activity in pKO-based agrobacteria lines. Tobacco leaves from independent plants (n=2) were infused with an agro-mixture containing 50% CF/SR and 50% of pIG121, sense (pKO-sFLUC), antisense (pKO- aFLUC), or hairpin (pKO-ihpFLUC) binary vectors. The average RLU from independent leaf discs assays (n=4) was determined 66 and 138 h after agroinfiltration. The CF/SR+pKO RLUs are expressed as a percentage of CF/SR+pIG121 activity ($RLU_{pKO}/RLU_{pIG121} \times 100$). pIG121 serves as a control binary vector. Error bars represent the standard error determined from the average of 66- and 138-h measurements. **(B)** RiLUC activity levels increase with increasing densities of agrobacteria harboring pKO-ihpFLUC in *Nicotiana benthamiana*. Tobacco leaves from independent plants (n=2) were infused with an agrobacteria line containing 25% CF/SR and 5%, 15%, 50%, and 75% of pKO-ihpFLUC. Agrobacteria harboring pIG121 were used to make the total amount of agrobacteria equal to 100%. RLUs were measured 66 and 138 h after agroinfusion. Error bars represent the standard error from individual leaf disc assays (n=2) from 2 separate leaf infusions (n=2).

hpRNA-mediated PTGS of the FiLUC gene reduces silencing of the coexpressed RiLUC gene

The cotransferred *Renilla* luciferase (RiLUC) reporter gene is usually used as an internal control to help normalize data between assays. When RiLUC activity was examined during assays of pKO-induced silencing of FiLUC, it was noted that the RiLUC values from CF/SR+pKO-ihpFLUC infusions were consistently elevated when compared with either the CF/SR+pIG121 control or the CF/SR+pKO-sFLUC (sense) and CF/SR+pKO-aFLUC (antisense) silencing assays (Figure 4A). These assays were performed by using an infusion of *N. tobaccum* and *N. benthamiana* leaves (duplicate independent plants) with a mixed agrobacteria culture containing 50% CF/SR and 50% of one the silencing (or control) constructs. The average of the 2 time point (66 and 138 h) measurements of RiLUC activity was determined with our leaf disc assay. For ease of comparison, RiLUC activities from the CF/SR+pKO-based constructs were calculated as a percentage of CF/SR+pIG121, ($[RLU_{pIG121} - RLU_{pKO}]/RLU_{pIG121} \times 100$, Figure 4A).

RiLUC activity levels were not significantly different in CF/SR+pKO-sFLUC or CF/SR+pKO-aFLUC infusions (~100%), relative to the CF/SR+pIG121 control. Surprisingly, a 2-fold increase in RiLUC activity levels was consistently observed in CF/SR+pKO-ihpFLUC (Figure 4A). To determine if this nonspecific suppression of silencing has a threshold, *N. benthamiana* leaves from duplicate independent plants were infused with agrobacteria-mixtures containing 25% CF/SR and 5%, 15%, 25%, 50%, or 75% of pKO-ihpFLUC. Agrobacteria harboring pIG121 were used to bring the total density of agrobacteria to 100%. *Renilla* luciferase activity was determined 66 and 138 h after infusion (Figure 4B). pKO-ihpFLUC–mediated suppression of RiLUC PTGS was observed by

using 5% of pKO-ihpFLUC and increased with higher agrobacteria concentrations of pKO-ihpFLUC. The increase in RiLUC activity (Figure 4B) observed with higher percentages of pKO-ihpFLUC harboring agrobacteria is inversely correlated with FiLUC activity (decreases with higher pKO-ihpFLUC, Figure 2B). Interestingly, 5% of pKO-ihpFLUC can effectively silence 90% of FiLUC activity levels, and 50% of pKO-ihpFLUC is sufficient to observe at least a 2-fold increase in RiLUC activity levels.

This is the first report, to our knowledge, that demonstrates cross-talk between RNA-mediated silencing of 2 unrelated (i.e., with no significant homology) reporter genes in plants. hpRNA-mediated interference of FiLUC reporter gene expression appears to measurably reduce PTGS of the coexpressed RiLUC reporter gene. Akashi and colleagues reported that in their BY2 cell culture system the dsRNA (inverted repeat of FLUC) was a strong inducer of PTGS of the FLUC gene but had no adverse effects upon expression of the sea pansy normalization gene (Akashi et al., 2001). Our agrobacteria-based transient expression system may be able to generate higher levels of dsRNA than those produced from electroporated culture cells, possibly overwhelming the PTGS system. Alternatively, PTGS within cultured tobacco cells may simply function somewhat differently from the silencing system active within intact leaf cells. In either case, it will be necessary to further explore the apparently passive suppression effect observed with hpRNA-mediated silencing.

Conclusion

pKO was found to be a very useful plasmid for dissecting PTGS and the cellular recognition of aberrant RNA transcripts in transient systems. hpRNA is a very effective initiator of PTGS; the exact mechanism of this trigger has yet to be clearly elucidated. The agrobacteria transient system and targeted pKO plasmids provide a rapid and relatively simple system for analysis of how viral suppressors reduce PTGS initiated by both functional (sense) and aberrant (hpRNA) transcripts. It will be interesting to further explore PTGS during transient expression by combined expression of viral suppressors (e.g., HcPro, p19) and plant genes involved in RNA-mediated silencing (e.g., DICER-like proteins). Finally, care should be taken when using co-infused reporter genes for signal normalization, especially when plasmid constructs that encode hpRNA are used in an *Agrobacterium* transient expression system.

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