Silencing on the Spot. Induction and Suppression of RNA Silencing in the *Agrobacterium*-Mediated Transient Expression System[1]

Lisa K. Johansen and James C. Carrington*

Institute of Biological Chemistry, Washington State University, Pullman, Washington 99164–6340

The *Agrobacterium*-mediated transient expression assay in intact tissues has emerged as a rapid and useful method to analyze genes and gene products in plants. In many cases, high levels of active protein can be produced without the need to produce transgenic plants. In this study, a series of tools were developed to enable strong or weak induction of RNA silencing and to suppress RNA silencing in the absence of stable transgenes. Transient delivery of a gene directing production of a double-stranded green fluorescent protein (GFP) transcript rapidly induced RNA silencing of a codelivered GFP reporter gene, effectively preventing accumulation of GFP protein and mRNA. RNA silencing triggered by the strong dsGFP inducer was partially inhibited by the tobacco etch virus silencing suppressor, P1/HC-Pro. In the absence of the strong double-stranded GFP inducer, the functional GFP gene served as a weak RNA silencing inducer in the transient assay, severely limiting accumulation of the GFP mRNA over time. The weak silencing induced by the GFP gene was suppressed by P1/HC-Pro. These results indicate RNA silencing can be triggered by a variety of inducers and analyzed entirely using transient gene delivery systems. They also indicate that RNA silencing may be a significant limitation to expression of genes in the *Agrobacterium*-mediated transient assay but that this limitation can be overcome by using RNA silencing suppressors.

RNA silencing in plants (also known as post-transcriptional gene silencing) is the remarkable process, whereby foreign RNA molecules are recognized and degraded in a sequence-specific manner (Meins, 2000; Sijen and Kooter, 2000). The foreign RNAs can derive from a highly expressed or aberrant transgene or from an infectious virus. In fact, RNA silencing is an adaptive defense response that can limit virus infection and the severity of symptoms (Marathe et al., 2000). RNA silencing in plants is closely related to the process of RNA interference in animals, which has been studied most intensively in *Caenorhabditis elegans* and *Drosophila* (Hunter, 2000). In many organisms, RNA silencing has proven to be a highly effective tool for producing epigenetic knockout phenotypes in whole organisms (Baulcombe, 1999; Bosher and Labouesse, 2000).

Through genetic and biochemical analyses in a variety of systems, the molecular basis for RNA silencing is partially understood (Bass, 2000; Carrington, 2000). A key early step in RNA silencing is formation of double-stranded (ds) RNA. In the case of most plant viruses, dsRNA is formed during the intermediate steps of genome replication, and this may explain why viruses are often potent inducers of RNA silencing (Baulcombe, 1999). RNA silencing triggered by transgenes, but not some viruses, requires an RNA-dependent RNA polymerase (RdRp)-like protein that is hypothesized to catalyze synthesis of RNA complementary to the target species (Dalmay et al., 2000; Mourrain et al., 2000). Double-stranded RNA is then recognized by a dsRNA-specific nuclease and cleaved to produce small (21–23 nucleotides) RNA species (Hamilton and Baulcombe, 1999; Hammond et al., 2000; Zamore et al., 2000). The small RNAs are proposed to associate with one or more nuclease-like proteins and serve as guides for sequence-specific cleavage of silencing target RNAs (Bass, 2000). This explains how a given inducer molecule can trigger RNA degradation directed against itself and against any RNA with high levels of sequence identity.

The differential requirements for RNA silencing triggered by transgenes and by RNA viruses in plants, and the effects of various virus-encoded silencing suppressors support a model in which there are two induction pathways leading to RNA silencing in plants (Carrington, 2000; Dalmay et al., 2000; Voinnet et al., 2000). Silencing triggered by a transgene mRNA, or an RNA with limited amounts of ds secondary structure, depend on the "weak" inducer pathway that involves the RdRp. It is interesting that this pathway also leads to systemic RNA silencing in which tissues distal to the initial sites of silencing induction also acquire the silenced state (Voinnet et al., 2000). Systemic silencing involves a graft-transmissible signal that moves through the phloem (Fagard and Vaucheret, 2000). Silencing triggered by some replicating RNA viruses, and possibly by inducers with very long segments of dsRNA, may occur through a "strong" inducer pathway in which the requirement for the RdRp is bypassed. The strong

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* Corresponding author; e-mail carrington@wsu.edu; fax 509–335–2482.

inducers may be recognized directly by the dsRNase required for synthesis of the small RNAs (Zamore et al., 2000).

The *Agrobacterium tumefaciens*-mediated transient expression system is a versatile tool to rapidly introduce genes into plant tissue. This system enables gene expression within a short period of time and without the requirement for regenerating transgenic plants. A useful feature of this system is the ability to introduce multiple genes simultaneously into a patch of leaf tissue. This system will likely increase in utility, particularly for high-throughput functional genomic and proteomic analyses. The *Agrobacterium*-mediated expression system has also been used effectively as a means to deliver RNA silencing inducers and suppressors into transgenic plants that express a silencing reporter gene (for example, Brigneti et al., 1998; Voinnet et al., 1998, 2000; Llave et al., 2000).

In this study, we developed tools and procedures to enable analysis of RNA silencing using the *Agrobacterium*-mediated transient expression system in the absence of a stable transgene reporter. The response of a reporter gene in the presence of weak and strong RNA silencing inducers was analyzed as was the effect of co-introduction of a virus-encoded silencing suppressor. The results indicate that highly effective RNA silencing can be triggered rapidly with strong inducers. The results also indicate that RNA silencing may be an inevitable consequence of *Agrobacterium*-mediated transient delivery of functional genes under the control of a strong promoter but that this can be countered through use of silencing suppressors.

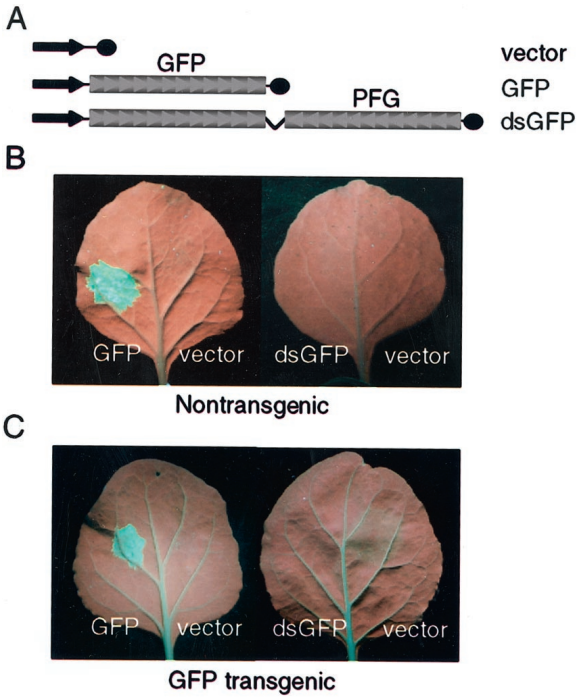

Figure 1. *Agrobacterium*-mediated transient expression in *N. benthamiana* leaves. A, Constructs used contained the 35S promoter (arrow) and terminator sequences (black circle). The GFP construct contained a functional copy of the soluble-modified GFP coding sequence, whereas the dsGFP construct contained both sense and antisense smGFP sequences separated by an intron. B and C, *Agrobacterium*-infiltrated non-transgenic (B) and GFP-transgenic (C) leaves were viewed at 6-d p.i. under long wavelength UV illumination. Spots in half leaves were infiltrated with *Agrobacterium* containing the indicated constructs.

RESULTS

Transient Delivery of RNA Silencing Inducers and Targets

Most studies to analyze RNA silencing in plants have depended on transgenic plants that express an active or silenced reporter gene. To enable analysis of RNA silencing that is independent of transgenes, an *Agrobacterium*-mediated transient system was devised to simultaneously introduce both silencing inducer and target RNAs in *Nicotiana benthamiana*. Two key constructs were used in most experiments. A 35S-green fluorescent protein (GFP) gene (referred to as the GFP construct) encoded the soluble-modified form of green fluorescent protein. A 35S-GFP/antisense GFP gene contained the full-length GFP coding sequence, an intron, and a full-length GFP sequence in the inverted orientation (Fig. 1A). Transcription of this gene and RNA processing was predicted to yield an intron spliced hairpin RNA that was referred to as the dsGFP RNA. Constructs directing synthesis of dsRNAs in transgenic plants were shown to be potent inducers of RNA silencing (Waterhouse et al., 1998; Chuang and Meyerowitz, 2000; Schweizer et al., 2000; Smith et al., 2000). An empty vector construct was also used in all experiments as a negative control.

Leaves of *N. benthamiana* plants were infiltrated with cultures of *Agrobacterium* containing vector, GFP, or dsGFP constructs, and GFP fluorescence was monitored using a handheld long wavelength UV light source. Noninfiltrated zones and zones infiltrated with cells containing the vector alone appeared red due to autofluorescence. Tissue infiltrated with bacteria containing the GFP gene appeared bright green (Fig. 1B). In contrast, tissue infiltrated with *Agrobacterium* containing the dsGFP construct appeared red and was indistinguishable from the vector-only infiltration sites. Similar results were obtained when *Agrobacterium* cultures containing the vector, GFP and dsGFP constructs were injected into leaves of GFP-expressing transgenic *N. benthamiana* plants. In these plants, GFP expressed from the injected construct was detected against a background of light green fluorescence from the transgene-expressed protein (Fig. 1C).

RNA Silencing by a Strong Inducer in the Transient System

A time-course analysis of GFP- and dsGFP-expressing tissue was done to examine the initiation of RNA silencing in the infiltrated tissues. High M_r and small RNAs were extracted from *Agrobacterium*-injected tissue and analyzed by blot hybridization with a radiolabeled probe specific for the GFP sequence. The small RNA fraction was prepared to analyze RNA silencing-associated 21- to 23-nucleotide RNA species. In cells undergoing RNA silencing, these small RNAs correspond to both sense and antisense fragments of the silencing target (Hamilton and Baulcombe, 1999). In non-transgenic tissue, the 35S-GFP mRNA was detected at 2-d postinfiltration (p.i.). After peaking at 3-d p.i., however, the level of GFP mRNA declined dramatically through 6-d p.i. (Fig. 2A). Relatively little small RNA with homology to the GFP sequence was detected during the time course. In contrast, neither a full-length dsGFP transcript nor a unit length GFP RNA was detected in non-transgenic tissue expressing the dsGFP gene (Fig. 2A). However, GFP-specific small RNA was detected at 2-d p.i. and accumulated over the 6-d time course in non-transgenic plants. In the GFP-expressing transgenic plants, the dsGFP gene also induced formation of GFP-related small RNAs as well as a moderate decline in the level of GFP transgene mRNA between 2- and 6-d p.i. (Fig. 2B). Transient expression of the dsGFP gene, therefore, was sufficient to induce RNA silencing in the injection zone of both non-transgenic and GFP-transgenic plants.

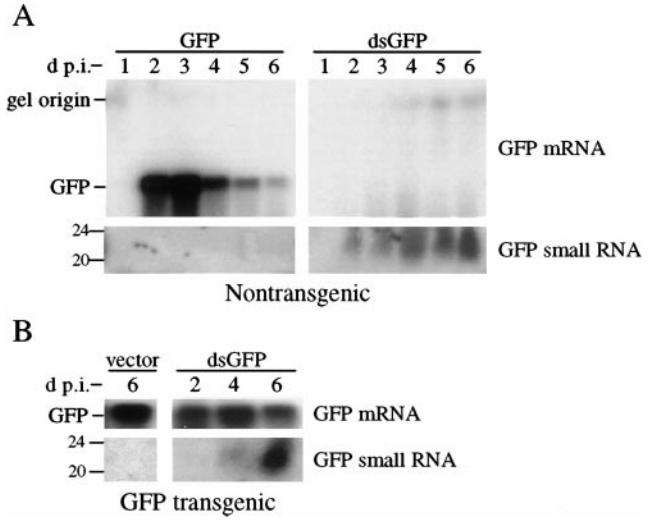

Figure 2. Analysis of GFP-related RNAs from tissue infiltrated with *Agrobacterium* containing the GFP and dsGFP genes. HMW RNA (5 μg) and small RNA (50 μg) samples were prepared at various times p.i. and subjected to RNA-blot analysis using a radiolabeled GFP sequence probe. A, Time course analysis of GFP-related RNAs in *Agrobacterium*-infiltrated non-transgenic leaf tissue expressing GFP or dsGFP genes. B, Limited time-course analysis of GFP-related RNAs in GFP-transgenic plants that were infiltrated with *Agrobacterium* containing empty vector or the dsGFP gene. The electrophoretic positions of GFP mRNA and oligonucleotide standards (20 and 24 nucleotides) are shown at the left.

To determine if transient dsGFP expression was sufficient to silence the *Agrobacterium*-injected GFP gene, coinfiltration experiments with both GFP and dsGFP constructs were done. In these and subsequent experiments, three *Agrobacterium* cultures were mixed in equal parts prior to all injections. One culture contained the GFP reporter gene. Depending on the experiment, the other two cultures contained empty vector, the dsGFP construct, or another test construct (see below). In all cases, however, the amount of injected *Agrobacterium* containing the GFP reporter was constant, regardless of whether or not additional cultures containing test constructs were added to the injection mix. In non-transgenic plants, infiltration of an *Agrobacterium* mixture containing the GFP gene and empty vector resulted in bright green fluorescence within 2 d of p.i. (Fig. 3, A and C). GFP fluorescence required a Vir^+ *Agrobacterium* strain, as tissue injected with a Vir^- strain containing the GFP construct failed to fluoresce (Fig. 3G). Infiltration of a mixture containing the GFP gene, dsGFP gene, and empty vector resulted in no GFP fluorescence (Fig. 3A). The same results were obtained when the mixtures were injected into GFP-expressing transgenic plants (Fig. 3, D and F). The suppression of GFP activity in tissue injected with the GFP plus dsGFP mixture required that the dsGFP construct be in a Vir^+ *Agrobacterium* strain (Fig. 3H). Furthermore, the GFP-inhibitory effect of the dsGFP construct was sequence-specific, as co-injection of *Agrobacterium* containing the GFP construct and a dsGUS construct resulted in strong GFP fluorescence (Fig. 3I).

The effect of co-introduction of GFP and dsGFP genes was investigated by analysis of GFP protein in the infiltrated tissue of transgenic and non-transgenic plants. As controls, tissues were infiltrated with *Agrobacterium* containing empty vector, GFP plus empty vector, and dsGFP plus empty vector. The GFP protein was detected in transgenic plants but not non-transgenic plants injected with *Agrobacterium* containing empty vector (Fig. 4, A and B, lanes 1–3). In non-transgenic tissue injected with the GFP gene plus empty vector, GFP protein accumulated to increasing levels over the 6-d time course (Fig. 4A, lanes 4–6), whereas in transgenic tissue GFP accumulated to levels higher than the endogenous (transgene-encoded) levels (Fig. 4B, compare lanes 4–6 with 1–3). However, in non-transgenic tissue infiltrated with the *Agrobacterium* mixture containing GFP and dsGFP genes, no GFP protein was detected at any time point (Fig. 4A, lanes 10–12). These data indicate that the dsGFP construct was inhibitory to accumulation of protein encoded by the injected GFP gene. Similarly, the levels of endogenous GFP in the transgenic plants decreased 5-fold, relative to tissue expressing the empty vector, over the time-course in

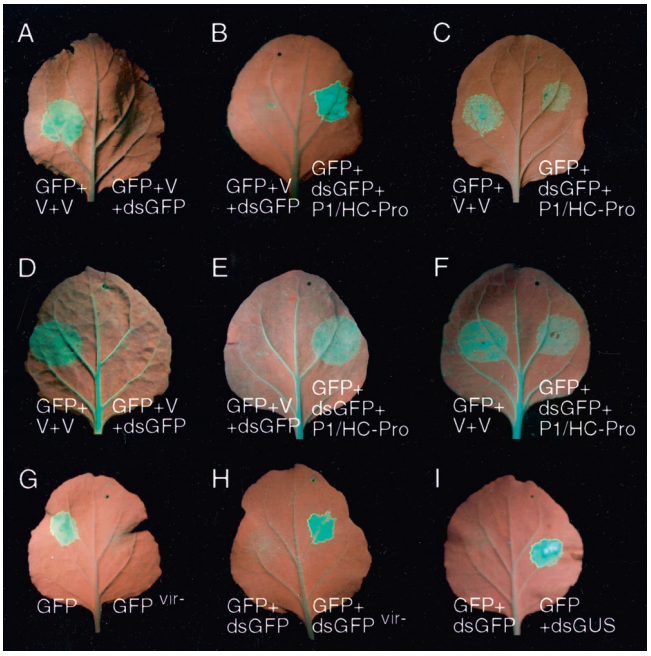

Figure 3. *Agrobacterium*-mediated transient expression of combinations of GFP, dsGFP, and P1/HC-Pro constructs. Non-transgenic (A–C and G–I) or GFP-transgenic (D–F) *N. benthamiana* leaves were infiltrated and analyzed as described in Figure 1. The vir− strain of *Agrobacterium* lacked DNA transfer properties and was used in a series of controls (G and H). All infiltrations with a mixture of three *Agrobacterium* cultures (A–F) were done using equivalent amounts of the individual components. In half leaves shown in A, C, D, and F, two equivalents of the empty vector (V) culture were used.

tissue infiltrated with the *Agrobacterium* mixtures containing the dsGFP gene alone or the dsGFP plus GFP genes (Fig. 4B, lanes 7–12).

Co-introduction of GFP and dsGFP genes was further investigated by analysis of GFP mRNA and small RNAs from the infiltrated tissues of non-transgenic plants. As in the previous experiment (Fig. 2A), infiltrated tissue expressing the GFP gene contained the GFP mRNA, which declined significantly between the 4- and 6-d-p.i. time points (Fig. 5, lanes 2–4). This decrease in GFP mRNA steady-state level over time contrasted with the increasing accumulation of GFP protein (Fig. 4A, lanes 4–6). The high steady-state level of protein likely resulted from the high stability of GFP. Little or no GFP-related small RNA was detected using these experimental conditions, even at 6-d p.i. (Fig. 5, lanes 2–4). However, these small RNAs were detected using higher specific activity probes and increasing exposure times (data not shown). No GFP mRNA was detected after co-introduction of the GFP and dsGFP genes, whereas small RNA accumulated to increasing levels throughout the time course (Fig. 5, lanes 8–10). The cumulative data from in situ visualization of fluorescence, and from analysis of GFP protein, GFP mRNA and GFP small RNA indicate that the dsGFP construct induced silencing rapidly and efficiently in the transient system, regardless of whether or not a homologous nuclear transgene was present.

Suppression of RNA Silencing by Tobacco Etch Virus (TEV) P1/HC-Pro in the Transient System

The TEV-encoded RNA silencing suppressor, P1/HC-Pro (Anandalakshmi et al., 1998; Brigneti et al., 1998; Kasschau and Carrington, 1998), was introduced into the GFP-based transient silencing system to address two issues. First, the ability of P1/HC-Pro to suppress RNA silencing from the "strong" inducer derived from the dsGFP gene was tested. In previous studies, P1/HC-Pro was shown to reverse RNA silencing triggered by a "weak" transgene inducer in transgenic plants (Llave et al., 2000). Second, the ability of P1/HC-Pro to inhibit decline of the GFP mRNA in tissues expressing the functional GFP gene was tested. If the decline was due to slow or weak induction of RNA silencing, then P1/HC-Pro was predicted to inhibit the decline. P1/HC-Pro is actually a polyprotein that undergoes autoproteolytic processing catalyzed by proteinase domains within the P1 and HC-Pro proteins (Carrington et al., 1990).

In contrast to the lack of GFP fluorescence in tissues injected with mixtures of *Agrobacterium* containing GFP plus dsGFP genes, tissues infiltrated with the triple mixture containing GFP, dsGFP, and P1/HC-Pro genes exhibited bright green fluorescence, regardless of whether the plants were non-transgenic or GFP-transgenic (Fig. 3, B, C, E, and F). In non-transgenic plants, the appearance of green fluor-

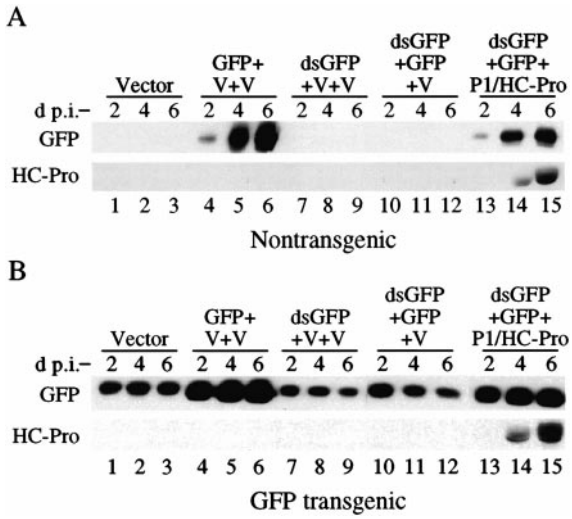

Figure 4. Immunoblot analysis of GFP and HC-Pro in *Agrobacterium*-infiltrated tissue. Time course analysis of GFP and HC-Pro protein in non-transgenic (A) or GFP transgenic (B) *N. benthamiana* plants infiltrated with *Agrobacterium* containing empty vector (lanes 1–3) or combinations of *Agrobacterium* containing empty vector (V), GFP, dsGFP, or P1/HC-Pro constructs (lanes 4–15). Normalized extracts (20 μg) were prepared at 2-, 4-, and 6-d p.i. and subjected to immunoblot analysis with anti-GFP or anti-HC-Pro sera.

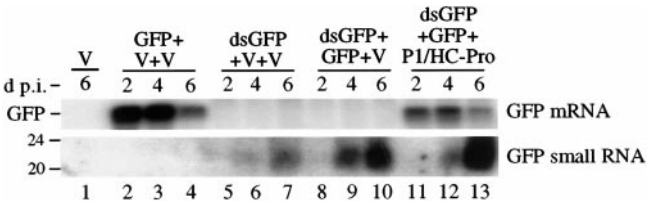

Figure 5. RNA-blot analysis of GFP-specific RNAs in *Agrobacterium*-infiltrated non-transgenic *N. benthamiana* tissue. HMW RNA (5 μg) and small RNA (50 μg) samples were prepared at various times p.i. and subjected to RNA-blot analysis using a radiolabeled GFP sequence probe. Samples were extracted from tissue that was infiltrated with *Agrobacterium* containing empty vector (V, lane 1) or combinations of *Agrobacterium* containing empty vector (v), GFP, dsGFP, or P1/HC-Pro constructs (lanes 2–13). The electrophoretic positions of oligonucleotide standards (20 and 24 nucleotides) are shown at the left.

escence in leaves injected with the triple mixture corresponded with accumulation of GFP protein in time-course experiments (Fig. 4A, lanes 13–15). HC-Pro was also detected in the immunoblot assay at 4- and 6-d p.i., although accumulation of HC-Pro was delayed relative to accumulation of GFP (Fig. 4A, lanes 13–15). In transgenic plants, the level of GFP protein that accumulated in tissues receiving the triple mixture was enhanced relative to tissues injected with the GFP plus dsGFP mixture (Fig. 4B, lanes 10–15). The enhancement (11.3-fold) was particularly evident at 6-d p.i. (Fig. 4B, compare lanes 12 and 15). In addition, the presence of P1/HC-Pro in non-transgenic tissues injected with the triple mixture resulted in accumulation of GFP mRNA (Fig. 5, lanes 11–13). However, as seen in tissues expressing GFP alone (Fig. 5, lanes 2–4), the GFP mRNA declined between 4- and 6-d p.i. Furthermore, the presence of P1/HC-Pro did not prevent accumulation of silencing-specific small RNAs (Fig. 5, lanes 11–13). At the 6-d p.i. time-point, in six independent experiments, the level of accumulation of GFP-related small RNAs in tissue injected with the triple mixture was equal to or greater than that detected in tissue expressing GFP plus dsGFP alone. These data suggest that P1/HC-Pro partially suppresses RNA silencing initiated by the strong inducer in the transient GFP expression system, although suppression likely occurs only at the early time points examined.

In addition to examination of the effect of P1/HC-Pro on dsGFP-induced RNA silencing, the effect of P1/HC-Pro on expression and accumulation of the GFP mRNA in the absence of dsGFP was tested. As in previous experiments (Figs. 2A and 5), tissue injected with *Agrobacterium* containing the GFP gene accumulated GFP mRNA to relatively high levels within 2-d p.i., but GFP mRNA levels declined at later time points (Fig. 6, lanes 1–3). In contrast, tissue injected with an *Agrobacterium* mixture containing GFP and P1/HC-Pro genes accumulated GFP mRNA to relatively high levels that did not decrease between 2- and 6-d p.i. (Fig. 6, lanes 10–12). These data indicate that the slow decline in GFP mRNA after *Agrobacterium*-mediated introduction of the GFP gene into leaf tissue is likely due to RNA silencing and that this is efficiently suppressed by P1/HC-Pro. This differs from the effect of P1/HC-Pro on dsGFP-mediated silencing of the GFP mRNA where the suppressor promotes accumulation of the GFP mRNA early (2-d p.i.) but not late (6-d p.i.) in the time course (Fig. 6, lanes 7–9).

DISCUSSION

RNA Silencing in the Transient Assay

The *Agrobacterium*-mediated transient expression system was used to deliver RNA silencing inducer, reporter, and suppressor constructs to intact tissues of *N. benthamiana*. This system enabled analysis of RNA silencing of a GFP construct based entirely on genes delivered in the transient assay. RNA silencing triggered by a strong inducer derived from the dsGFP construct occurred rapidly, regardless of whether or not the plant contained a GFP transgene. A key feature of this system is the ability to simultaneously introduce additional genes along with silencing reporter genes. For example, the effects of silencing suppressors can be tested by adding *Agrobacterium* cultures containing test constructs to the injection mix. The codelivery of multiple constructs is enabled by the extremely high efficiency of *Agrobacterium*-mediated gene transfer in *N. benthamiana* leaves. Microscopic examination of tissue injected with *Agrobacterium* containing the GFP construct suggests that virtually all cells express the gene (unpublished observations).

The finding that transient delivery of dsGFP triggered RNA silencing efficiently is fully consistent with several other studies using transgenic plants (Waterhouse et al., 1998; Chuang and Meyerowitz, 2000; Smith et al., 2000). The basis for this strong inducer activity relates to the probable role of dsRNA as the substrate for a nuclease that catalyzes cleavage to 21 to 23 nucleotide RNAs (Zamore et al., 2000). The

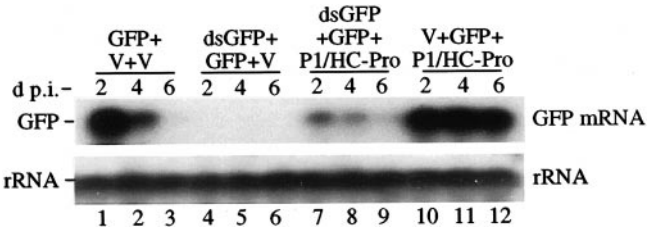

Figure 6. RNA-blot analysis of GFP-specific RNAs in *Agrobacterium*-infiltrated non-transgenic *N. benthamiana* tissue. HMW RNA samples (5 μg) were prepared at various times p.i. and subjected to RNA-blot analysis using a radiolabeled GFP sequence probe. Samples were extracted from tissue that was infiltrated with combinations of *Agrobacterium* containing empty vector (V), GFP, dsGFP, or P1/HC-Pro constructs. The blot was stripped and reprobed using radiolabeled DNA corresponding to ribosomal RNA.

small RNAs are proposed to guide a nucleolytic ribonucleprotein complex to target RNAs. Thus, increasing amounts of dsRNA would lead to increasing amounts of small RNAs, which would lead to increasing amounts of a component of the sequence-specific nuclease. In clear support of this model, small RNAs were produced in relatively high quantities after introduction of the dsGFP construct. This was in stark contrast to the relatively low levels of small RNA in tissues expressing the functional GFP gene, at least during the time course examined.

Despite the relatively low levels of small RNA in the functional GFP-expressing tissue, RNA silencing was eventually detected in the absence of the dsGFP inducer. Similarly, Voinnet et al. (2000) found that infiltration of a GFP-expressing construct in GFP transgenic plants resulted intitally in strong fluorecence at the site of infiltration, followed by systemic silencing of the GFP transgene and small RNA accumulation. The results of experiments using P1/HC-Pro support the hypothesis that the decline in GFP steady-state level was due to RNA silencing. Co-introduction of GFP and P1/HC-Pro constructs resulted in maintenance of relatively high steady-state levels of GFP mRNA. As P1/HC-Pro has little or no effect on transcription (Kasschau and Carrington, 2001), maintenance of high GFP mRNA levels by P1/HC-Pro likely resulted from RNA silencing suppression.

These studies underscore the idea that there are two types of RNA silencing inducers (Dalmay et al., 2000; Voinnet et al., 2000). Strong inducers are those that contain extensive amounts of dsRNA, either because a gene directs synthesis of a transcript that adopts considerable double stranded structure or because a replicating virus produces dsRNA during the course of genome replication. Weak inducers are those that contain relatively little double stranded structure but that are eventually recognized by the silencing apparatus and targeted. An important step in targeting a weak inducer may be recognition by the cellular RdRp, which is proposed to catalyze synthesis of complementary RNA and which would lead to accumulation of dsRNA intermediates (Dalmay et al., 2000; Mourrain et al., 2000). How a weak inducer is initially recognized remains to be determined.

The effects of TEV P1/HC-Pro on RNA silencing induced by a β-glucuronidase (GUS) transgene was analyzed previously (Kasschau and Carrington, 1998; Llave et al., 2000). The GUS transgene in those studies likely resulted in formation of a weak inducer RNA in transgenic plants. Co-expression of P1/HC-Pro effectively suppressed both RNA silencing and formation of small RNAs (Kasschau and Carrington, 1998; Llave et al., 2000). In the dsGFP-induced system described here, P1/HC-Pro transiently suppressed RNA silencing induced by the dsGFP construct. However, RNA silencing triggered by the dsGFP construct in the presence of P1/HC-Pro eventually occurred in the 6-d time course experiments, resulting in declining GFP mRNA levels and accumulation of small RNAs. It is proposed that in the presence of low levels of dsRNA inducer, P1/HC-Pro effectively suppresses RNA silencing. However, as dsRNA inducer accumulates, the suppressing activity of P1/HC-Pro is overcome, and RNA silencing occurs. These data suggest that P1/HC-Pro inhibits a dsRNA-dependent step in the RNA silencing pathway.

Applications

There are three types of applications that arise from this work. First, the transient RNA silencing assay using various types of inducers and GFP as a reporter provides a rapid method to screen candidate genes, or random genes from a library, for effects of RNA silencing. Screens could be designed for positive or enhancing effects in the case of genes encoding RNA silencing activators or effectors. Alternatively, screens can be done for silencing suppressors that have a negative effect. Such a strategy for identification of positive and negative factors involved in RNA silencing will complement mutant screens.

Second, the transient delivery of dsRNA constructs provides a rapid method to potentially silence any gene in the *Agrobacterium* infiltration zone. Of course, the use of dsRNA-mediated transient silencing depends on the availability of an assay to monitor effects, and the range of processes that might be investigated using this approach in leaf tissue is limited. The transient RNA silencing system is further limited by potential residual effects of gene products that accumulate prior to induction of silencing. This point is illustrated by examination of the effects of dsGFP on endogenous GFP protein and mRNA levels in GFP-transgenic tissue (Figs. 2B and 4B). Although the dsGFP inducer triggered RNA silencing, residual GFP protein and GFP mRNA were still detected at 6-d p.i. In addition, the applicability of the transient system is limited to those species that are amenable to delivery and expression of T-DNA constucts by *Agrobacterium*.

Third, the effect of P1/HC-Pro on RNA silencing triggered by functional (weak silencing inducer) genes has broad use. The use of the *Agrobacterium* delivery system to introduce foreign genes into leaf tissue continues to grow. Further, we see tremendous potential for this system in functional genomics and proteomics programs, in which expression of wild-type or tagged proteins is followed by analysis of effects on global gene expression, metabolic pathways, or protein complex formation. Under the control of the 35S promoter, it is clear that the GFP gene is subject to RNA silencing after an initial burst of gene expression. It is reasonable to expect that many

genes, expressed in a similar manner, will follow the same pattern. Co-introduction of P1/HC-Pro with the GFP gene suppressed the RNA silencing response. It follows, therefore, that P1/HC-Pro will suppress RNA silencing triggered by other constructs and result in maintenance of high expression levels for extended periods.

MATERIALS AND METHODS

Plasmid Construction

The base vector for all constructs, pRTL2 (Restrepo et al., 1990), contained an enhanced 35S promoter from cauliflower mosaic virus, the TEV 5′-non-translated sequence, and the 35S terminator.

The GFP construct (pRTL2-smGFP) contained the coding region of the soluble-modified green fluorescent protein from *Aequorea victoria* (nucleotides 21–737) (Davis and Vierstra, 1998). An additional codon (GCA) was inserted immediately after the start codon to form a *Nco*I restriction site at the 5′ end of the open reading frame. The 3′ end of the GFP coding sequence contained the authentic stop codon followed by a *Kpn*I site. The GFP coding sequence was inserted between the *Nco*I and *Kpn*I sites of pRTL2.

The dsGFP construct (pRTL2-dsGFP) contained the entire GFP open reading frame, including the stop codon, a 120-nucleotide intron from the *RTM1* gene of Arabidopsis Col-0 (Chisholm et al., 2000), and the entire GFP coding region in the antisense orientation. The dsGFP construct was made by joining the *RTM1* intron to the 3′ end of the GFP coding sequence using PCR. This fragment was cloned into pRTL2-smGFP using *Kpn*I and *Xba*I restriction sites.

The dsGUS construct (pRTL2-dsGUS) contained the entire GUS coding region, followed by the *RTM1* intron, and then an antisense copy of the 3′-proximal 558 nucleotides of the GUS coding sequence. The intron-antisense GUS fragment was inserted into pRTL2-GUS (Restrepo et al., 1990) between the *Bgl*II and *Bam*HI sites.

Construction of the P1/HC-Pro construct (pRTL2–0027) was described previously (Carrington et al., 1990). This construct contained the sequence corresponding to nucleotides 12 to 2,681 of the TEV genome, which encodes the P1 and HC-Pro proteins and the N-terminal 82 amino acid residues of the P3 protein.

The expression cassette from each pRTL2-based construct was excised using *Pst*I and inserted into the plant transformation vector, pSLJ755I5 (Jones et al., 1992). Each of these plasmids was introduced into *Agrobacterium tumefaciens* strain GV2260 or the avirulent strain C58C1^{D} by triparental mating.

Plant Material and *Agrobacterium* Infiltration

Transgenic *Nicotiana benthamiana* plants expressing GFP protein were provided by Dr. David C. Baulcombe (Sainsbury Laboratory) and were described previously (Schaad et al., 1997; Brigneti et al., 1998). *Agrobacterium* infiltration of leaves was done as described (Llave et al., 2000) except that the cultures were incubated overnight in infiltration medium at room temperature. *Agrobacterium* cultures were mixed prior to infiltration by combining equal volumes of individual cultures. A 3-cc syringe was used to infiltrate tissue from the underside of leaves.

GFP Imaging

Visual detection of GFP fluorescence was done using a long-wave UV lamp (Black Ray model B 100 AP). Plants were photographed with a 950 digital camera (Nikon, Tokyo) mounted with both UV and yellow filters. The images were processed electronically using Adobe Photoshop.

RNA Isolation and Blot Analysis

Total RNA from infiltrated spots was extracted by grinding leaf tissue in liquid nitrogen and resuspending the frozen powder in Trizol reagent (10 [v/w]) (Life Technologies/Gibco-BRL, Cleveland). After 5 min at room temperature, chloroform was added (0.2 [v/v]) and the solution was mixed thoroughly. The RNA was separated from the DNA and protein by centrifugation at 12,000*g* for 15 min at 4°C. The RNA phase was removed, and RNA was precipitated by the addition of isopropanol (0.5 [v/v]). The RNA pellet was dissolved in 1 mL of Qiagen buffer QRL1 (Qia RNA/DNA Midi Kit). Nine milliliters of QRV2 buffer was added to the solution. The RNA was applied to a Qiagen RNA/DNA column according to the manufacturer's directions. Low M_r (LMW) RNA was eluted with buffer QRW2, and high M_r (HMW) RNA was subsequently eluted with buffer QRU. The RNA was precipitated with ice-cold isopropanol (1 [v/v]) and recovered by centrifugation at 15,000*g* for 30 min at 4°C. The RNA pellets were resuspended in diethyl pyrocarbonate-treated water, and total RNA concentration was determined using a UV-1601 spectrophotometer (Shimadzu, Columbia, MD).

The LMW RNA (50 μg) was resolved by electrophoresis in a 15% (w/v) polyacrylamide-7 M urea gel in TBE buffer (45 mM Tris-borate, pH 8.0, 1 mM EDTA). The HMW RNA (5 μg) was resolved by electrophoresis in a 1.5% (w/v) agarose-formaldehyde gel using a buffer consisting of 20 mM HEPES, pH 7.8, 1 mM EDTA. The RNA in gels was transferred to HyBond-N membrane and subjected to UV crosslinking (1,200 μJ, Stratalinker, Stratagene, La Jolla, CA). The LMW and HMW RNA blots were prehybridized in solution (50% formamide [v/v], 10× Denhardt's solution, 0.5 mg/mL sheared salmon sperm DNA, 1% [w/v] SDS, 3× SSC, and 50 mM phosphate buffer) at 35°C and 42°C, respectively, for at least 3 h. GFP specific radioactive DNA probes were generated by a random priming technique. Hybridization of the LMW (35°C) and HMW (42°C) blots was done overnight in a rotating incubator and was followed by four washes (20 min each) in 2× SSC buffer and 0.2% (w/v) SDS at 50°C and 65°C, respectively. Radio-

activity on the blots was quantitated using a phosphoimager. Blots were then exposed to x-ray film.

Immunoblot Analysis

Leaf tissue from infiltration zones was ground in liquid nitrogen and resuspended (5 [v/w]) in dissociation buffer (40 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.1% [v/v] Triton X-100, 0.1% [w/v] *N*-lauryl sarcosine, 10 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, and 1 μg/mL leupeptin). Total protein concentration was determined by the method of Bradford using the Protein Assay dye reagent (Bio-Rad Laboratories, Hercules, CA). Protein samples (20 μg) were subjected to SDS-PAGE and immunoblot analysis using anti-GFP (Promega, Madison, WI) or anti-HC-Pro-specific sera. Immunoreactions were detected using an alkaline phosphataselinked second antibody and a chemiluminescence procedure. Blots were exposed to x-ray film for different periods of time. Densitometry of bands was done using an Eagle Eye II system (Stratagene).

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