Appl Biochem Biotechnol (2009) 158:243–252
DOI 10.1007/s12010-008-8332-y

A Viral Suppressor P1/HC-Pro Increases the GFP Gene Expression in *Agrobacterium*-mediated Transient Assay

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Received: 9 May 2008 / Accepted: 24 July 2008 /
Published online: 13 August 2008

Abstract More than 20 post-transcriptional gene silencing (PTGS) suppressors have been found since HC-Pro, the first gene silencing suppressor, was found in 1998. The silencing suppressor strongly suggested that gene silencing functions as natural defense mechanisms against viruses. It also represented a valuable tool for the dissection of the gene silencing pathway. We have used P1/HC-Pro RNA silencing suppressor activity to increase green fluorescent protein (GFP) expression in tobacco using an *Agrobacterium*-mediated transient expression system. P1/HC-Pro stimulated GFP-gene expression but not dsGFP-gene expression was shown by RT-PCR, Northern and Western blot analysis. Expression of the gene silencing suppressor and the target gene provided a new strategy of heterogeneous gene expressing in plants. It may be of commercial significance to produce foreign proteins using plant bioreactors.

Keywords Post-transcriptional gene silencing (PTGS) · Suppressor · P1/HC-Pro · *Agrobacterium*-mediated transient expression system · GFP · Semi-quantitative reverse transcription-PCR

Abbreviations

HC-Pro	helper component-proteinase
PTGS	post-transcriptional gene silencing
RISC	RNA-induced silencing complex
RT-PCR	reverse transcription-PCR
siRNAs	short-interfering RNAs

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Introduction

Recently the field of RNA silencing in plants, animals, protozoa, and a plant fungal pathogen has intensely investigated for fundamental reasons and also because of its potential application for crop improvement [1–4]. In higher plants, post-transcriptional gene silencing (PTGS) functions as a natural anti-viral defense because plant viruses are both initiators and targets of PTGS, and also encode proteins that are PTGS suppressors [5–10]. A key early step in RNA silencing is the formation of double-stranded (ds) RNA which can be introduced experimentally or arise from endogenous transposons, replicating RNA viruses, or the transcription of transgenes [11]. The dsRNA is then cleaved by a ribonuclease III (RNAse III)-like enzyme, termed Dicer, into 21–24 nucleotide duplexes (short-interfering RNAs (siRNAs)) [12–14]. The siRNAs are denatured and incorporated into a multi-subunit endonuclease silencing complex called the RNA-induced silencing complex (RISC) [15]. Within the activated RISC, single-stranded siRNAs act as the guide to bring the complex into contact with complementary mRNAs and thereby cause their degradation [12, 14–17]. In plants, RNA silencing of transgenes is typically associated with the methylation of nuclear DNA corresponding to the transcribed region of the target RNA, although transcription level of the transgene is generally unaffected [18]. A fascinating feature of RNA silencing in plants is that after initiation of local silencing, a signal is produced that can move between cells through plasmodesmata and over long distance through the vascular system to direct specific RNA silencing in the whole plant [19–23].

Some plant viruses encode proteins with anti-silencing activity, which allow them to evade silencing [6, 9, 24]. Different PTGS-suppressing proteins appear to act at different points in the PTGS pathway [23, 25]. The potyviruses encode helper component-proteinase (HC-Pro), a multifunctional protein required for maintenance of genome replication, long-distance movement through plants and polyprotein processing [26–28]. HC-Pro was the first identified suppressor of RNA silencing. The original reports demonstrated that it suppressed both transgene- and virus-induced silencing [29–30]. Although HC-Pro alone has suppressor activity [25, 29], it is frequently expressed as the proteinase 1 (P1)/HC-Pro polyprotein construct, which is the N-terminal portion of the natural viral polyprotein and allows HC-Pro to be proteolytically processed as it is expressed from the viral genome. Some evidence suggests that P1 might enhance HC-Pro activity as a suppressor of silencing [31]. Recently CMV2b [32], P25 [23], Cys-rich 16 kD protein [33], P14 and P15 [34], NSs [35], rb [36], P0 [37], P19 [38–39], NS3 [40], p21 and p22 [41], P38 [42], C2 [43], AC2 and AC4 [44], Pns10 [45], and CP [42, 46] have been reported as gene silencing suppressors besides HC-Pro. These findings will shed light on the mechanism of gene silencing.

In this study, we demonstrate that P1/HC-Pro is able to increase the level of green fluorescent protein (GFP) expression in the *Agrobacterium*-mediated transient expression system.

Materials and Methods

Plasmid Construction

The GFP construct (pSLJ75515-smGFP) contained the coding region of soluble-modified green fluorescent protein from *Aequorea Victoria* (nucleotides 21–737) [47].

The dsGFP construct (pSLJ75515-dsGFP) contained the entire GFP open reading frame, including the stop codon, a 120-nucleotide intron from the *RTM1* gene of Arabidopsis Col-0 [48], and the entire GFP coding region in the antisense orientation.

The P1/HC-Pro construct (pSLJ75515-0027) was described previously [49]. This construct contained the sequence corresponding to nucleotides 12 to 2,681 of the TEV genome, which encoded the P1 and HC-Pro proteins and the N-terminal 82 amino acid residues of the P3 protein.

The expression cassette from each pSLJ-based construct was excised using *Pst*I and inserted into the plant transformation vector, pCAMBIA1301. Each of these plasmids was introduced into *Agrobacterium tumefacien* GV2260 by a freeze-thaw method.

Plant Materials and *Agrobacterium* Infiltration

Wild-type *Nicotiana benthamiana* was grown as previously described [21, 50, 51]. *Agrobacterium* infiltration of leaves was performed according to Voinnet et al., 1998 [21]. For co-infiltration, equal volume of each *Agrobacterium* cultures (OD_{600}=1) were mixed before infiltration. A 5-ml syringe was used to infiltrate leaves from the underside.

GFP Imaging

GFP fluorescence was visually detected using a 100 W, long-wave UV lamp (Black Ray model B-100 AP; Ultra-Violet Products, Upland, CA, USA). Plants were photographed with a digital camera (Sony DSC-F828) mounted with both UV and yellow filters. The images were processed electronically using Adobe Photoshop.

Semi-quantitative Reverse Transcription-PCR

RNA from infiltrated leaf spots was extracted using RNAgents® Total RNA Isolation System Kit (Z5110, Promega) according to the instructions provided by the manufacturer. The quality and concentration of RNA preparations were accurately determined with a UV-visible spectrophotometer (model $DU^{(R)}$ 640; Beckman, USA). The DNase-treated samples of total RNA (1 μg) were used in the first-strand cDNA synthesis reaction with the reagents supplied in the reverse transcription system (Promega, A3500), according to the manufacturer's instruction. The RNA samples were primed with oligo $(dT)_{15}$, and the reverse transcriptase reaction was performed at 42 °C for 30 min, 95 °C for 5 min, and 4 °C for 5 min in a final volume of 20 μl. The first-strand cDNA synthesis reagents were diluted to 100 μl with TE buffer or nuclease-free water. Samples from each reaction containing the single-stranded cDNA template (10–20 μl) were used in a 100 μl polymerase chain reaction (PCR) mixture of final composition: 10 mM dNTP mixture, 25 mM $MgCl_2$, 10× Taq polymerase buffer (Promega), and 2.5 units Taq DNA Polymerase (Promega). GFP cDNA was amplified using two primers (P1: 5'-ATGAGTAAAGGAGAAGAACTTTT-3' and P2: 5'-TTATTTGTATAGTTCATCCATGC-3'). PCR was performed for 30 cycles (94 °C, 40 s; 51 °C, 40 s; 72 °C, 40 s), followed by 72 °C for 10 min to produce a 716 bp fragment. P1/HC-Pro cDNA was generated by PCR with two primers (P3: 5'-TAAAGGCAATA AACTAACAGGGGC-3' and P4: 5'-TCCCTCCAACATTGTAAGTTTTCAT-3'). PCR was conducted in 30 cycles (94 °C, 40 s; 52 °C, 40 s; 72 °C, 40 s), followed by 72 °C for 10 min to produce a 953 bp fragment.

As a control, the 400 bp fragment of tobacco actin gene was amplified by reverse transcription-PCR (RT-PCR) using two primers (P5: 5'-GCAACTGGGATGATATGGAG-3'

and P6: 5'-TCACGTGTAAGCGAGTTTTTC-3'). PCR was performed for 30 cycles (94 °C, 40 s; 52 °C, 40 s; 72 °C, 45 s). These PCR products were separated on 1% agarose gels and visualized by ethidium bromide staining. Gels were scanned in a UV image instrument (UVIPhotoMW, BTS-20.M, EEC) and the individual band relative intensity (semi-quantitative) value assigned by UVISofe-UVITech, UK, 1999 software, respectively.

Northern Blot Analysis

Samples of 10 μg of total RNA were separated by electrophoresis in a 1.2% (*w*/*v*) agarose-formaldehyde gel (2.2 mol/L formaldehyde final) and blotted onto a nylon membrane. For probe labeling, the *Gene Image*™ Random Prime Labeling module was used (Amersham Life Science, UK). Labeled fluorescent probes were hybridized to blotted RNA, which was pre-hybridized and hybridized at 65 °C overnight. The hybridization buffer contained 5% SSC, 0.1% sodium dodecyl sulfate (SDS), and 20-fold dilution of the liquid block. The membrane with blotted RNA was then washed at 65 °C using a 1% SSC and 0.1% SDS solution followed by incubation in a liquid blocking solution as recommended by the supplier. Membranes were then incubated with a 5,000-fold dilution of anti-fluorescein-alkaline-phosphatase-conjugated antibody to obtain a fluorescent signal. After washing, fluorescent signals on the membrane were detected using a *Gene Images*™ CDP-Star detection module (Amersham Life Science, UK). The membranes were finally wrapped in a protective film and exposed to an X-ray film.

Immunoblot Analysis

Leaf tissues from the infiltration area were ground in liquid nitrogen and resuspended (2 (*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 phenylmethylsulfony fluoride, 1 μg/mL aprotinin, and 1 μg/mL leupeptin). Protein amount and purity were determined by Bradford assay [52]. Protein samples (20 μg) were subjected to SDS-PAGE and immunoblot analysis using anti-GFP antibody (Sigma). The SuperSignal® WestPico Trial Kit and the manufacturer's protocol were used in the detection of GFP. The individual band relative intensity value assigned by UVISofe-UVITech, UK, 1999 software.

Results

Phenotypes in Infected Plants

In *N. benthamiana* plants examined under UV illumination, non-infiltrated areas and areas infiltrated with *Agrobacterium* cells containing the vector alone, appeared red due to autofluorescence (Fig. 1a). Tissues infiltrated with an *Agrobacterium* mixture containing the GFP gene and vector alone showed the bright green GFP fluorescence (Fig. 1b). In contrast, tissues infiltrated with an *Agrobacterium* mixture containing the dsGFP gene and the vector alone appeared red (Fig. 1b) and were indistinguishable from the areas infiltrated with the vector alone. GFP fluorescence was not apparent in tissues infiltrated with the mixture of *Agrobacterium* containing the GFP gene, dsGFP gene, and vector alone. However tissues infiltrated with a mixture containing the P1/HC-Pro genes in addition to either GFP and dsGFP, or the GFP gene and vector alone, exhibited bright green

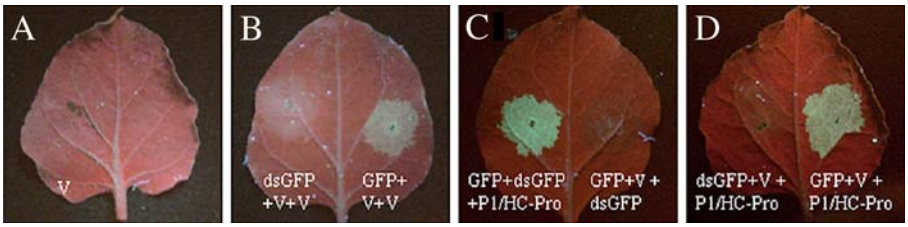

Fig. 1 *Agrobacterium*-mediated transient expression of GFP, dsGFP, and P1/HC-Pro in *N. benthamiana* leaves. All infiltrations with a mixture of three *Agrobacterium* cultures (**b**–**d**) were performed using equivalent amounts of the individual components. In leaves shown in **b** double amounts of empty vector (*V*) culture were used whereas in **a** only empty vector culture (*V*) were used in half leaves (marked by *V*)

fluorescence (Fig. 1c,d). In contrast, when the GFP gene was omitted, an infiltration mixture containing the P1/HC-Pro gene, dsGFP gene, and vector alone resulted in no GFP fluorescence (Fig. 1d).

Analysis of RNA Level in Infected Plants

We performed semi-quantitative RT-PCR to analyze the expression of GFP and P1/HC-Pro. An actin gene fragment (400 bp) was amplified from each sample examined as the control. The intensities of the amplified actin were the same, indicating that an equal amount of total RNA of each sample was used for RT-PCR amplification. The time course showed that GFP expression increased from 1.5- to 6-days post-infiltration (dpi) in tissues of *N. benthamiana* injected with an *Agrobacterium* mixture containing the GFP gene and vector alone (Fig. 2, 2–4). However, in tissues injected with an *Agrobacterium* mixture containing the GFP gene, dsGFP gene, and vector alone, the GFP expression level declined from 1.5 to 6 dpi. No GFP cDNA was detected at 6 dpi (Fig. 2, 8–10). In contrast the expression of GFP was increased from 1.5 to 6 dpi when the P1/HC-Pro gene was included in the *Agrobacterium* injection mixtures containing either the GFP gene, dsGFP gene, or the GFP gene vector alone (Fig. 2, 11–16). Furthermore, the inclusion of the P1/HC-Pro gene in these mixtures increased the GFP expression at 6 dpi GFP relative to tissues injected with an *Agrobacterium* mixture containing the GFP gene and vector alone. cDNA was not detected in tissues injected with an *Agrobacterium* containing the vector alone or an *Agrobacterium* mixture containing the dsGFP gene and vector alone or an *Agrobacterium* mixture containing the dsGFP gene, P1/HC-Pro gene, and vector alone (Fig. 2, 1, 5–7, 17–19). The expression level of P1/HC-Pro gene increased from 1.5 to 6 dpi in tissues injected with an *Agrobacterium* mixture containing the P1/HC-Pro gene (Fig. 2, 11–19).

A similar result was obtained by Northern hybridization analysis. At 6 dpi the band corresponding to the GFP gene was more intense in tissues injected with all *Agrobacterium* mixtures containing the P1/HC-Pro gene except that with the dsGFP and vector alone (Fig. 3). In this case there were no GFP bands at 6 dpi in tissues injected with a mixture, containing the vector alone, the dsGFP gene and the vector alone (Fig. 3). At 6 dpi bands were seen corresponding to the P1/HC-Pro gene in tissues injected with all *Agrobacterium* mixtures containing the P1/HC-Pro gene (Fig. 3).

Protein Analysis in Infected Plants

GFP protein was detected by Western blot analysis at higher levels (>threefolds) in tissues of *N. benthamiana* injected with *Agrobacterium* mixture containing the GFP gene,

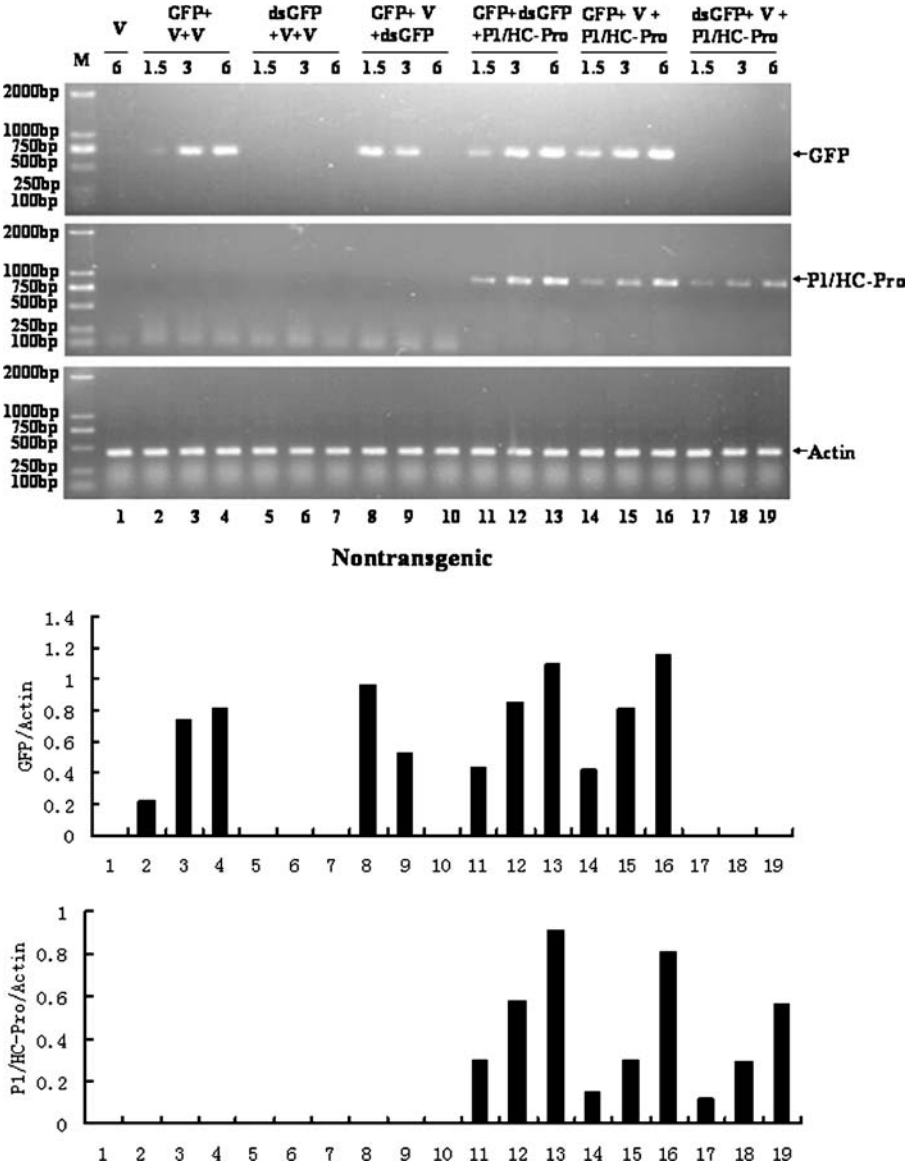

Fig. 2 Semi-quantitative RT-PCR analysis of GFP and P1/HC-Pro in *Agrobacterium*-infiltrated tissues. Analysis of GFP, P1/HC-Pro and Actin expression at 1.5, 3 and 6 days post-infiltration of *N. benthamiana* with *Agrobacterium* containing vector alone (lane *1*) or combinations of *Agrobacterium* containing vector alone (V), GFP, dsGFP, or P1/HC-Pro constructs (lanes *2–19*)

P1/HC-Pro gene, and the vector alone or the GFP gene, dsGFP gene, and P1/HC-Pro gene than those injected with an *Agrobacterium* mixture containing the GFP gene and the vector alone at 6 dpi (Fig. 4). Three experiments were performed with the similar results.

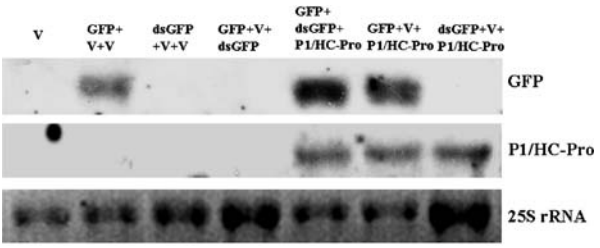

Fig. 3 Northern hybridization analysis of GFP and P1/HC-Pro in *Agrobacterium*-infiltrated tissues. Total RNAs were prepared from *N. benthamiana* at 6 dpi. Samples were extracted from tissues that were infiltrated with combinations of *Agrobacterium* containing empty (V), GFP, dsGFP, or P1/HC-Pro constructs. Total RNAs loaded in each lane (10 μg) were visualized by Ethidium bromide (EtBr)-staining. 25S rRNA is shown as a loading control

Discussion

In recent years, plant genetic engineering has dramatically increased the production of a variety of molecules of nutritional, therapeutic, and industrial importance. Favorable prospects, including production costs, safety, and environmental issues indicate that plants may become the preferred recombinant protein bioreactor [53]. However, specific transgene construct expression varies considerably within populations of transgenic plants. This variation hampers proper evaluation of transgene and is undesirable during high-throughput transgene screening. Various factors are thought to contribute to variation in transgene expression including the transgene copy number, RNA silencing, transgene insertion site, and the employment of certain regulatory sequences to drive transgene expression [54]. The p19 protein (P19) of tomato bushy stunt virus is a pathogenicity determinant with host-dependent effects on virus spread and symptom induction. In addition, p19 is a suppressor of PTGS, preventing incorporation of siRNA into RISC. With p19 GFP co-expression was

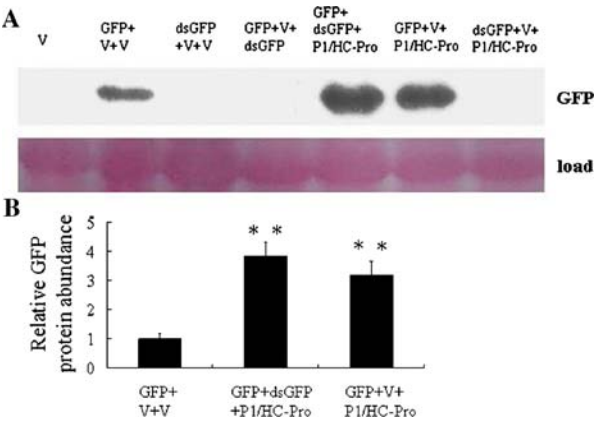

Fig. 4 GFP protein analysis in infected plants. **a** Immunoblot analysis of GFP in *Agrobacterium*-infiltrated tissues. Samples were extracted from tissues infiltrated with combinations of *Agrobacterium* containing empty (V), GFP, dsGFP, or P1/HC-Pro constructs. A total of 20 μg extracts for each sample were prepared at 6-dpi and subjected to immunoblot analysis with anti-GFP sera. Ponceau S staining of total proteins indicated equal loading. **b** Densitometric analysis of immunoblot. All GFP protein levels, normalized to total protein, were expressed relative to the GFP expression alone. Three experiments were performed with similar results. Data are means±SEM (n=3), **p<0.01 vs. control group

enhanced 50-fold or more [38]. P1/HC-Pro is also a suppressor of PTGS, targeting a maintenance step of the RNA silencing pathway downstream to the signal production but upstream to the production of the 25 nt RNAs [55, 56]. In this study GFP production was enhanced threefold or more in the presence of P1/HC-Pro. The results indicated that P1/HC-Pro was able to increase the level of GFP expression in an *Agrobacterium*-mediated transient expression system.

Co-expression of a transgene and a viral suppressor gene might be an attractive option to reduce variation of transgene expression caused by RNA silencing. Co-expression of the gene silencing suppressor and the target gene provides a new strategy of heterogeneous gene expression in plants. It can be commercially useful for the production of heterologous proteins using the plant bioreactor.

Acknowledgement This work was supported by the Program for Changjiang Scholars and Innovative Research Team (PCSIRT) in University (#IRT0519), the Programme of Introducing Talents of Discipline to Universities 111 Project B07017 and the Chinese National Plant Transformation Center (JY03-B-17). We thank Dr. Carrington, Institute of Biological Chemistry, Washington State University, for providing pSLJ75515-smGFP, pSLJ75515-dsGFP and pSLJ75515-0027 constructs; Dr. Baulcombe, the Sainsbury Lab, UK, for *N. benthamiana*.

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