Biotechnol Lett (2007) 29:641–645
DOI 10.1007/s10529-006-9279-0

ORIGINAL RESEARCH PAPER

Transient co-expression of post-transcriptional gene silencing suppressors and β-glucuronidase in harvested lettuce leaf tissue does not improve recombinant protein accumulation *in planta*

Christopher W. Simmons · Jean S. VanderGheynst

Received: 22 November 2006 / Accepted: 29 November 2006 / Published online: 6 January 2007

Abstract *Agrobacterium*-mediated gene transfer was used to co-express three virus-derived post-transcriptional gene silencing (PTGS) suppressors, P19 from tomato bushy stunt virus and two species of helper component proteinase (HcPro) from tobacco etch virus (TEV) and turnip mosaic virus, with β-glucuronidase (GUS) in harvested lettuce leaf tissue to investigate whether GUS accumulation increases in the presence of PTGS suppressors. Co-expression incubations were 3–5 days at 4 and 22°C. GUS activity and leaf viability were measured after incubation. Co-expression of PTGS suppressors did not elevate GUS expression levels. Under certain incubation conditions, co-expression of TEV HcPro significantly lowered transient GUS expression and was detrimental to leaf viability, suggesting that expression of PTGS silencers may have a negative effect on transient expression levels that outweighs any effects of PTGS suppression in harvested leaf tissues.

Keywords Agroinfiltration · post-transcriptional Gene silencing · Transient expression

C. W. Simmons · J. S. VanderGheynst (✉)
Department of Biological and Agricultural Engineering, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA
e-mail: jsvander@ucdavis.edu

Introduction

Post-transcriptional gene silencing (PTGS) is a highly conserved, plant-immune response thought to have arisen as a defense against plant viruses. Moreover, any inserted gene that exhibits abhorrently high expression may trigger post-transcriptional silencing in plant cells (Matzke et al. 1995). Given that the expression of many inserted transgenes in plants is driven by viral promoters, post-transcriptional gene silencing would appear to be a logical candidate for impeding transient transgene expression.

Through co-evolution, viruses have adapted a variety of post-transcriptional gene silencing suppressors (Anandalakshmi et al. 1998), which can take inhibitory action at several points in the silencing pathway. Co-expression of these silencing suppressors along with reporter genes has shown that silencing suppressors are effective at inhibiting PTGS and elevating transient expression of the reporter genes in agroinfiltrated tobacco plants (Llave et al. 2000; Voinnet et al. 2003).

While PTGS clearly has a negative effect on transient expression in tobacco, it is unknown whether PTGS has any role in inhibiting transient expression in harvested tissues. Research has shown that transient expression levels in harvested agroinfiltrated lettuce are time dependent and taper off approximately 72 h after

agroinfiltration (Joh et al. 2005), but it is unclear if this decrease in expression is due to the plant's PTGS response or other factors.

To ascertain whether PTGS was responsible for the leveling of transient expression in lettuce and if expression levels could benefit from the presence of silencing suppressors, the viral PTGS suppressors P19 from tomato bushy stunt virus (TBSV) and two species of helper component proteinase (HcPro) from tobacco etch virus (TEV) and turnip mosaic virus (TuMV) were co-expressed in lettuce along with the reporter gene for β-glucuronidase (GUS) via *Agrobacterium*-mediated gene transfer. TBSV has been shown to be infectious towards lettuce (Liu et al. 1999; Obermeier et al. 2001), making P19 an ideal candidate for suppressing PTGS. TEV and TuMV are potyviruses, which are virulent towards a variety of dicots (Reichmann et al. 1992), suggesting their species of HcPro may be able to arrest PTGS as well.

Materials and methods

Agrobacterium tumefaciens strains and cultivation

Agrobacterium tumefaciens strain C58 was used to harbor the binary T-DNA vectors. C58 employs a binary vector system in which all the of the virulence genes necessary for T-DNA transfer to host plant cells are contained on a helper plasmid (Hamilton 1997) while the T-DNA is contained on a separate vector. Table 1 describes the various vectors, T-DNA-encoded genes and selection criteria used in cultivation.

All strains of *A. tumefaciens* were grown in pH 7.0 media, which consisted of 10 g Bacto-peptone/l, 10 g yeast extract/l and 5 mg NaCl/l (YEP) supplemented with selection components as listed in Table 1. The flasks were grown overnight at 28°C and shaken at 150 rpm. Prior to agroinfiltration cultures were centrifuged at 4,000 *g* for 20 min at 10°C. Cell pellets were resuspended in sterile distilled water to a final absorbance of 0.35 at 590 nm (OD_{590} 0.35) on a microplate reader.

Plating and colony forming unit (c.f.u.) quantification

To determine if differences existed in cell concentrations at a given optical density, liquid cultures of each strain were grown to an OD_{590} of approximately 0.35 and then diluted $1{:}10^6$ in sterile distilled water. Diluted cell suspensions were then plated on to solid YEP (YEP media with 10 g agarose/l) containing appropriate antibiotics. After incubation at 29°C for 36 h the colonies were counted. A cell dry weight to c.f.u. conversion factor of 0.21 pg dw/c.f.u. was determined by measuring the residual dry mass of 100 ml of cell suspension with a known c.f.u. density after heating overnight at 101°C.

Agroinfiltration

Hearts of Romaine lettuce, purchased from a commercial supplier, were washed in distilled water and discs of 1 cm diameter were cut from

Table 1 Vectors and constructs used in C58. Host plasmid information from (Wroblewski et al. 2005)

Strain	Vector or construct	Host plasmid	Protein encoded by gene expressed *in planta* under CaMV 35S promoter	Selection criteria in growth media
C58C1	pTFS40	pSLJ1006	GUS	5 mg tetracycline/l 50 mg kanamycin/l
C58 TEV HcPro	TEV-HcPro	pSLJ75515	TEV HcPro	5 mg tetracycline/l
C58 TuMV HcPro	TuMV-HcPro	pCB301	TuMV HcPro	5 mg tetracycline/l
C58 P19	P19	N/A	P19	5 mg tetracycline/l 100 mg spectinomycin/l
C58	none	pCB301	none	5 mg tetracycline/l

the mid-section of the leaves using a cork borer. Effort was made to avoid the midrib and select tissue of uniform color and texture, as described elsewhere (Joh et al. 2005).

To examine differences in GUS expression when GUS is co-expressed with PTGS suppressors, various cell suspension mixtures were prepared and agroinfiltrated into leaf tissue. This was accomplished by mixing equal volumes of 0.35 OD_{590} C58C1 cell suspension with 0.35 OD_{590} cell suspension containing a PTGS suppressor construct. In addition, C58C1 cell suspension was mixed with C58 as a control to measure the expression of GUS in the absence of suppressors while maintaining the same C58C1 and overall *A. tumefaciens* concentrations present in the other treatments. All mixtures and agroinfiltrations were performed in triplicate.

For each treatment, 20 leaf discs were added to 50 ml *A. tumefaciens* mixture in a 250 ml Erlenmeyer flask. Break-Thru S 240 (Goldschmidt Chemical, Hopewell, VA) surfactant was added to give 1 μl/l. The pressure within the flasks was then lowered to 25 kPa while being shaken at 70 rpm for 20 min. The vacuum was then rapidly broken. The leaf discs were removed from the flasks and placed in 100 × 15 mm Petri dishes lined with filter paper. The filter paper was wetted with 1 ml sterile water and then the dish was sealed with Parafilm. The plates were incubated in the dark at 4 or 22°C for 72 to 120 h. Leaves were examined for putrefaction and GUS expression after incubation. Leaves were deemed putrefied if they had decomposed during incubation.

Leaf protein extraction

Leaf disc samples were mechanically lysed by grinding in a chilled mortar and proteins extracted using a buffer consisting of 48 mM NaH_2PO_4/Na_2HPO_4 (pH 7.5) and 40 mM dithiothreitol (DTT) added at 1:1 (v/w). The crude extracts were transferred to 1.5 ml microcentrifuge tubes and centrifuged twice at 19,000 *g* for 20 min at 10°C. The supernatant portions were stored at –80°C until analysis.

Spectrophotometric GUS assay

GUS activity within the leaf extracts was measured using a spectrophotometric assay that measures the rate of *p*-nitrophenolate (PNP^-) formation as GUS cleaves glucuronic acid from *p*-nitrophenyl glucuronide (PNPG). Reaction mixtures included 50 μl leaf extract supernatant in 500 μl assay buffer consisting of 50 mM sodium phosphate buffer pH 7.5, 5 mM dithiothreitol, 0.1% (w/v) sodium lauryl sarcosine, 0.1% (v/v) Triton-X, 1 mM EDTA, and 6 mM PNPG (Sigma) at 37°C (Joh 2005). The formation of PNP^- was then monitored at 405 nm. The concentration of PNP^- was calculated through Beer's Law using a molar extinction coefficient of 18,700 l/mol cm for PNP^-. One enzyme activity unit, U, was defined as the formation 1 nmol product per min.

Data analysis

The statistical analysis program JMP IN v 5.1 (SAS Institute, Cary, NC) was used to perform a 2-way ANOVA and Tukey's honest squares difference (HSD) test on CFU concentration data and GUS expression levels.

Results and discussion

Each strain of *A. tumefaciens* had similar cell dry mass concentrations for the optical densities tested (Table 2). The cell concentrations are similar to those reported by Joh (2005) for C58C1 at OD_{590} 0.35. A pair-wise comparison of means revealed that there were no significant (alpha = 0.05) differences between the cell densities of each strain for cultures with an OD_{590} near 0.35. This observation demonstrates that a constant cell concentration would be achieved when equal parts of cultures from different strains are mixed to create various co-expression mixtures, given that all cultures have an OD_{590} of 0.35.

The GUS expression results at 3 days post-infiltration (dpi) for each co-expression treatment are shown in Table 3. A pair-wise comparison of means determined that there were no significant

Table 2 Mean optical density and corresponding cell dry weight densities for different strains of *A. tumefaciens*

Strain	Mean OD_{590} (SEM)[a]	Mean g dry wt/ml ($\times 10^{-4}$) (SEM)[a]
C58 TuMV HcPro	0.37 (0.007) A	2.31 (0.15) A
C58 P19	0.36 (0.016) A	2.49 (0.10) A
C58 TEV HcPro	0.36 (0.006) A	2.14 (0.15) A
C58	0.35 (0.004) A	2.18 (0.37) A

[a] $n = 4$, means not connected by the same letter are significantly different at alpha = 0.05

Table 3 β-glucuronidase expression in the presence of post-transcriptional gene silencing suppressors 3 days post-infiltration at an incubation temperature of 22°C

Proteins expressed *in planta*	Mean expression level (SEM) (U/g wet wt leaf)[a]
GUS	950 (94) A
GUS + P19	1011 (42) A
GUS + TEV HcPro	1177 (46) A
GUS + TuMV HcPro	1054 (76) A

[a] $n = 3$, means not connected by the same letter are significantly different at alpha = 0.05

differences (alpha = 0.05) in expression between any of the treatments. This result suggests that at 3 dpi PTGS does not influence GUS expression levels in harvested lettuce. Despite the absence of a statistically significant increase in expression, there appeared to be a slight elevation in GUS expression when GUS was co-expressed with TEV HcPro. To ascertain if GUS expression was, in fact, higher in the presence of TEV HcPro, the post-infiltration incubation period was extended and the incubation temperature was varied in an attempt to make any differences in expression due to PTGS suppression more pronounced.

A screening experiment was performed in which leaves were infiltrated with one mixture of C58C1 and C58 and another mixture of C58C1 and C58 TEV HcPro and subjected to two incubation lengths, 3 days and 5 days, and two incubation temperatures for the 5 day incubations, 4 and 22°C. Table 4 displays the expression results for each treatment. Several of the leaves corresponding to the GUS and TEV HcPro treatment with 5 days of incubation at 22°C underwent extensive putrefaction. As a result, they could not be assayed for GUS activity. Considering the data for the remaining treatments, which exhibited no putrefaction, when GUS was expressed alone for 5 days at 4°C the GUS expression levels were significantly higher than when co-expressed with TEV HcPro for 3 days at 22°C or 5 days at 4°C. These results agree with the implications of the prior experiment–specifically, that the presence of PTGS suppressors did not enhance GUS expression. On the contrary, these results suggest that co-expression of GUS with PTGS suppressors had a deleterious effect on overall GUS expression in harvested tissue, especially as incubation time and temperature increased. This is evidenced by the fact that treatments where leaves were agroinfiltrated with C58C1 and empty C58 had significantly higher GUS expression at 5 dpi compared to those agroinfiltrated with C58C1 and C58-TEV HcPro. Furthermore, the putrefaction of leaves co-expressing GUS and TEV HcPro 5 dpi at 22 °C, when compared to the complete lack of putrefaction in those solely expressing GUS

Table 4 β-Glucuronidase expression level when expressed alone and in the presence of tobacco etch virus HcPro for varying incubation durations and temperatures

Treatment	Mean number of putrefied leaf discs (SEM)[a]	Mean GUS expression level (SEM) (U/g wet wt/leaf)[a]
GUS 3 days at 22°C	0 (0) A	1380 (125) AB
GUS 5 days at 22°C	0 (0) A	1153 (94) AB
GUS 5 days at 4°C	0 (0) A	1432 (164) A
GUS + TEV HcPro 3 days at 22°C	0 (0) A	936 (43) B
GUS + TEV HcPro 5 days at 22°C	7 (1) B	N/A
GUS + TEV HcPro 5 days at 4°C	0 (0) A	926 (39) B

[a] $n = 3$, means within columns not connected by the same letter are significantly different at alpha = 0.05

under the same conditions, also indicated a negative effect of PTGS suppressor expression on leaf viability. There are reports of developmental abnormalities in transgenic plants expressing silencing suppressors that may potentially prove fatal to the host plant. This arises from the fact that the silencing suppressors may act upon PTGS components that are shared among other pathways not related to the plant's immune response (Takeda et al. 2005). The unregulated, constitutive expression of PTGS silencers within plant tissue may have enhanced these detrimental effects and played a role in the putrefaction of leaves transiently expressing HcPro after 5 days of incubation and the lack of a significant difference among treatments.

Acknowledgments We thank Dr. R. Michelmore and Dr. T. Wroblewski for supplying the *A. tumefaciens* strains used in this study.

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