Acta Biochimica Polonica

Vol. 53 No. 2/2006, 289–298

on-line at: www.actabp.pl

Regular paper

Optimization of transient *Agrobacterium*-mediated gene expression system in leaves of *Nicotiana benthamiana*

Mateusz Wydro[1,2], Edward Kozubek[1] and Przemysław Lehmann[1]✉

[1]*Institute of Plant Genetics, Polish Academy of Sciences, Poznań, Poland;* [2]*School of Neurology, Neurobiology and Psychiatry, The Medical School, University of Newcastle upon Tyne, UK;*
✉*e-mail: pleh@igr.poznan.pl*

Received: 26 October, 2005; revised: 09 February, 2006; accepted: 21 March, 2006
available on-line: 03 April, 2006

Here we report on a simple and reproducible system of *Agrobacterium*-mediated transient gene expression assay that utilizes infiltration of young *Nicotiana benthamiana* leaves. Although some of the phenomena described in this paper have been already reported by other researchers, here we have further developed them. The highest level of transient *gfp* gene expression was detected in the youngest leaves of *N. benthamiana* infiltrated with *A. tumefaciens* strains AGL0 and EHA105 precultured in the presence of 450–600 μM acetosyringone. Although the maximum level of transient *gfp* gene expression was restricted presumably by RNA silencing, it was completely suppressed in the presence of the viral protein HC-Pro. The transient expression system described here can be used to identify new viral suppressors of RNA silencing, for detailed analysis of unidentified genes and for industrial production of proteins in plants as well.

Keywords: *Agrobacterium*-mediated transient expression, RNA silencing, viral suppressor, *Nicotiana benthamiana*, green fluorescent protein (GFP), HC-Pro protein

INTRODUCTION

Agrobacterium-mediated transient gene expression system in intact plant leaves is a rapid and useful method of analysis of gene expression. By this method many different heterologous proteins can be produced without the need to generate transgenic plants, which is difficult for many plant species (Fischer *et al.,* 1999; 2004; Horn *et al.,* 2004). The *Agrobacterium*-mediated transient gene expression system is often used for identification of new viral suppressors of RNA silencing and for functional analysis of unidentified genes (Qu *et al.,* 2003; Thomas *et al.,* 2003). Transient gene expression system offers a number of advantages over stable expression. One of the most important advantages of the transient gene expression technique as compared to the stable gene expression is its simplicity and easy performance. Transient gene expression can be assayed directly post-agroinfiltration (Kapila *et al.,* 1997).

Usually *Agrobacterium*-mediated transient expression of a transgene achieves the highest level 2–3 days following argoinfiltration, after which the expression level decreases rapidly. This observation can be explained by triggering of local RNA silencing which blocks expression of transgenes (Johansen & Carrington, 2001; Voinnet *et al.,* 2003). RNA silencing is one of the major mechanisms of defense against viruses and other nucleic acid invaders in plants (Dougherty & Parks, 1995; Lindbo *et al.,* 1993; Smith *et al.,* 1994; English *et al.,* 1996; Baulcombe, 1996; 2004; Al-Kaff *et al.,* 1998; Tenllado *et al.,* 2004). Specific viral proteins able to suppress the RNA silencing mechanism have been identified in many plant viruses (Roth *et al.,* 2004). The plant viral suppressor proteins are expected to play im-

Abbreviations: AS, acetosyringone; GFP, green fluorescent protein; HC-Pro, helper component proteinase; *hptII,* hygromycin phosphotransferase; MES, morpholineethanesulfonic acid; *mgfp5,* modified green fluorescent protein gene; M-MLV, *Moloney murine leukemia virus*; P1, potyviral protein 1; TuMV, *Turnip mosaic virus*; PVY, *Potato virus Y*; siRNA, short interfering RNA; RT-PCR, Reverse Transcription-Polymerase Chain Reaction; TBSV, *Tomato bushy stunt virus*.

portant roles in the study of the mechanism of RNA silencing and as a tool for industrial production of proteins in plants. The molecular mechanism of suppression of RNA silencing has been fully described only for two viral suppressors: protein p21 encoded by the beet western yellow virus (Reed *et al.,* 2003) and p19 encoded by the tomato bushy stunt virus (TBSV) (Lakatos *et al.,* 2004). Both proteins (p21 and p19) bind to siRNA targeted to viral RNA and block siRNA interaction with its target — the specific viral RNA (Baulcombe & Molnár, 2004). A potyviral protein called helper component proteinase (HC-Pro) was one of the first identified suppressors of RNA silencing (Kasschau & Carrington, 1998; Anandalakshmi *et al.,* 1998). In contrast to the p21 and p19 suppressors, the molecular mechanism of HC-Pro activity is yet to be described. Many transient and stable transformation approaches have been used to explain the HC-Pro suppression of RNA silencing. HC-Pro influences the siRNA metabolism and alters the accumulation of various classes of siRNA (Mallory *et al.,* 2002; Hamilton *et al.,* 2002).

In this paper, we report on a modified *Agrobacterium*-mediated transient expression analysis based on suppression of gene silencing (Voinnet *et al.,* 2003) by efficient pre-induction of *Agrobacterium tumefaciens vir* genes. The green fluorescent protein (*gfp*) gene was used as a sensitive and non-invasive marker to visualize transient gene expression in transformed cells. This transient expression system can be used for identification of new viral suppressors of RNA silencing, for detailed analysis of unidentified genes and for industrial production of proteins in plants as well.

MATERIALS AND METHODS

Plant material. Wild-type *Nicotiana benthamiana* plants were grown under standard greenhouse conditions. For all assays 5- to 8-week-old *N. benthamiana* plants were used.

Bacterial strains and binary plasmids. *Agrobacterium tumefaciens* strains EHA105, LBA4404, AGL0 and AGL1 harboring binary vectors were used for transient transformation. Plasmid pCAMBIA(gfp)1302 (Center for the Application of Molecular Biology of International Agriculture, Canberra, Australia) contains hygromycin phosphotransferase (*hptII*) gene and a modified green fluorescent protein (*mgfp5*) gene. Plasmid pBINmgfp5-ER (Haseloff *et al.,* 1997) was kindly provided by Jacek Augustyniak (Adam Mickiewicz University, Poznań, Poland). All constructs used in this study are listed in Fig. 1.

Construction of suppressor-vectors. Total RNA was extracted with TRIZOL (GibcoBRL) from leaves infected with TuMV or PVY viruses and RT-PCR reactions were carried out. The incubation mixture for the reverse transcription reaction was based on the method given in the GeneAmp RNA PCR kit (Roche). In our routine experiments we used 50 units of reverse transcriptase (M-MLV), 25 μM random primer and 1 μg of total RNA as a template in the incubation mixture to synthesize the first-strand cDNA. After incubation at 99°C for 5 min, 2.5 units of AmpliTaq DNA polymerase and two primers were added to the reverse transcriptase mixture and then the PCR reaction was performed. To obtain the P1 and HC-Pro genes for preparation of the construct pJR1Ri-P1/HC-Pro(TuMV), the following two primers were used: NPL50 5′ AATCGGTACCAACAATGGCAGCAGTAACATTCGCA 3′ and NPL43ASC 5′ ATCATCATCATCGTCGACTCATCCGACGCGGTAGTGTTTCAAGC 3′ with sequences corresponding to the P1 and HC-Pro coding region of TuMV virus. For preparation of the construct pJR1Ri-HC-Pro(TuMV) the following two primers were used: NPL45A 5′ ATCGGTACCAACAATGAGTGCGGCAGGAGCCAATTTCTGG 3′ and

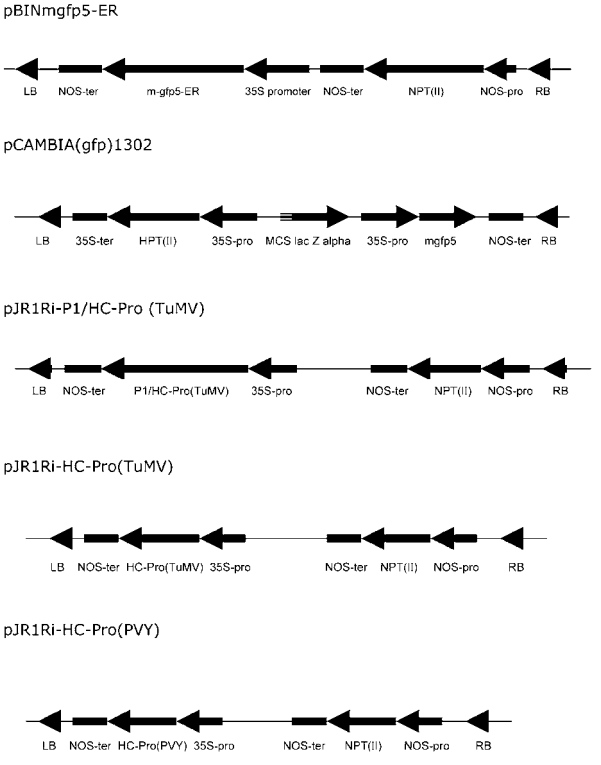

Figure 1. Schematic representation of the T-DNA region from the constructs used in the present study.
LB, left border; NOS-ter, terminator 3′NOS; m-gfp5-ER, modified *gfp* gene with HDEL peptide; NPT(II), neomycine phosphotransferase gene; NOS-pro, promoter NOS; RB, right border; 35S-ter, terminator 35S; HPT(II), hygromycin phosphotransferase gene; 35S-pro, promoter 35S; MCS, molecular cloning sites; lacZ alpha, β-galactosidase gene; mgfp5, modified *gfp* gene; P1+HC-Pro(TuMV), P1 and HC-Pro genes of TuMV virus; HC-Pro(TuMV), HC-Pro gene of TuMV virus; HC-Pro(PVY), HC-Pro gene of PVY virus.

NPL43ASC 5′ ATCATCATCATCGTCGACTCATCCGACGCGGTAGTGTTTCAAGC 3′ with sequences corresponding to HC-Pro of TuMV virus. For preparation of the construct pJR1Ri-HC-Pro(PVY) the following two primers were used: NPL50B 5′ ATCGGTACCAACAATGGCCAGCTTGCCAGTTAGCGATCTG 3′ and NPL43BSC 5′ ATCATCATCATCGTCGACTCATATGCCAGGGGATAGTATTGATA 3′. All the cloning steps were carried out using standard molecular biology protocols (Sambrook & Russell, 2001). DNA fragments from PCR reactions were gel-purified and ligated into expression vector pJR1Ri (kindly provided by Andrew Greenland, ZENECA SEEDS, Jealott's Hill Research Station, Bracknell, UK). All three constructs contained all the essential elements: left and right border regions, cauliflower mosaic 35S promoter, a pUC polylinker, 3′ termination region and the plant-selectable *npt(II)* gene. The above constructs contained the PCR-amplified fragment of viral cDNA carrying the HC-Pro gene or P1/HC-Pro that were cloned at the restriction sites: *Kpn*I and *Sal*I. The genes contained the start codon (ATG) and a good translation start context (AACA) (Kozak, 1984). These constructs (Fig. 1) were used to suppress the silencing during transient expressing of the *gfp* gene. All constructs were mobilized from *Escherichia coli* DH5α into *Agrobacterium tumefaciens* (four strains) containing helper plasmids using direct transformation of *Agrobacterium. A. tumefaciens* colonies capable of growing in a kanamycin-containing medium were selected for use in these studies.

Preparation of *Agrobacterium* cultures for agroinfiltration. The constructs used in the present study (pBINmgfp5-ER, pCAMBIA(gfp)1302, pJR1Ri-P1/HC-Pro(TuMV), pJR1Ri-HC-Pro(PVY), pJR1Ri-HC-Pro(TuMV)) were introduced into *A. tumefaciens* strain EHA105 (unless stated otherwise) by direct transformation. Recombinant *A. tumefaciens* were grown overnight at 28°C in 100 ml conical flask containing 10 ml of LB medium supplemented with 50 μg kanamycin per ml. Aliquote of 50 μl of this overnight culture was used for inoculation of 10 ml of LB medium supplemented with 10 mM MES buffer, pH 5.7, 50 μg kanamycin per ml and 150 μM acetosyringone (3,5-dimethoxy-4′-hydroxy-acetophenone) (Voinnet *et al.*, 2003). The precultures were grown overnight at 28°C in a shaker. Cells were harvested by centrifugation and resuspended to a final concentration corresponding to an optical density (OD) of 1.0 (Voinnet *et al.*, 2000) at 600 nm in a solution containing 10 mM $MgCl_2$, 10 mM MES pH 5.7, and 150 μM acetosyringone (unless stated otherwise). Cultures were incubated at room temperature for 3 h before infiltration.

Agroinfiltration procedure. For silencing-release assays, two zones on the opposite half-leaves of *N. benthamiana* plants were infiltrated with a mix of cultures from two recombinant *Agrobacterium* strains: the first culture carrying a Ti-binary plasmid that contained the *gfp* gene and the second culture carrying a Ti-binary plasmid in which a transgene encoding a viral suppressor was inserted. A mix two cultures of *Agrobacterium* carrying the Ti-binary plasmid pJR1Ri (i.e., empty vector) and a Ti-binary plasmid that contained the *gfp* gene was used as a control. Prior to infiltration a suspension of *Agrobacterium* harbouring a binary plasmid containing the *gfp* gene was mixed in a 1:1 ratio with a bacterial suspension carrying one of the three viral silencing suppressors in the binary plasmid. Two top-leaves per plant were infiltrated with a 2-ml syringe without a needle. Leaves were superficially wounded with a needle to improve infiltration. Three plants were agroinfiltrated for each point of a particular chart. The infiltrated leaves were photographed at 4 days after agroinfiltration (unless stated otherwise).

GFP imaging. Visual detection of GFP fluorescence in whole transient transformed leaves was performed using a hand-held long-wavelength ultraviolet lamp. The transiently transformed leaves were photographed with a Digital Camera OLYMPUS, C-750 Ultra Zoom though a Yellow 8, ES 52 filter (exposure time 16 s). The fluorescence intensity of randomly selected three sites in the agroinfiltrated and nonagroinfiltrated zones (as a background control) was measured in the photographs of *Agrobacterium*-infected leaves. The fluorescence was quantified using the ImageJ program (National Institutes of Health, USA) and fluorescence intensity was expressed as the mean gray value (Maximova *et al.*, 1998). The data were statistically analyzed by the *t*-test.

RESULTS

Comparison of levels of transient *gfp* gene expression in leaves of *N. benthamiana* mediated by four strains of *A. tumefaciens* carrying binary plasmids

For comparative purposes, the four strains (EHA105, AGL0, AGL1 and LBA4404) of *A. tumefaciens* carrying the binary plasmids pCAMBIA(gfp)1302 and pJR1Ri-HC-Pro(TuMV) were infiltrated into leaves of *N. benthamiana*. Suspensions of particular strains of *A. tumefaciens* carrying plasmid pCAMBIA(gfp)1302 were infiltrated separately or in combination with a culture carrying plasmid pJR1Ri-HC-Pro into leaves of *N. benthamiana.* The infiltrated leaves were photographed on the 4th day after agroinfiltration.

The highest level of GFP fluorescence without a suppressor was reached with strain AGL0 (about 30 as the mean gray value). The maximal level for strain EHA105 was 14 only. When strain AGL0 carrying pCAMBIA(gfp)1302 was used for agroinfiltration with the suppressor HC-Pro(TuMV) the level of GFP fluorescence was increased twice in relation to that in the absence of this suppressor (from 30 to 60 as the mean gray value). When strain EHA105 was used the GFP fluorescence was increased three times as compared to that without a suppressor (from about 14 to 40) (Fig. 2). We used interchangeably two bacterial strains: AGL0 and EHA105 in our experiments. When strains LBA4404 and AGL1 carrying plasmid pCAMBIA(gfp)1302 were used for agroinfiltration with HC-Pro(TuMV) the increase of the GFP fluorescence in relation to that without a suppressor was very poor (Fig. 2).

Comparison of levels of transient *gfp* gene expression in leaves of *N. benthamiana* mediated by *Agrobacterium* EHA105 carrying the binary plasmids pBINmgfp5-ER or pCAMBIA(gfp)1302

Suspensions of strain EHA105 carrying the binary plasmids: pBINmgfp5-ER, pCAMBIA(gfp)1302 or pJR1Ri-HC-Pro(TuMV) were infiltrated into leaves of *N. benthamiana*. Our results indicated that a higher level of GFP fluorescence was reached when the binary plasmid pBINmgfp5-ER was used (about 65 as the mean gray value with the suppressor and 35 without). When the plasmid pCAMBIA(gfp)1302 was used these values were 28 and 13, respectively (Fig. 3). Plasmid pBINmgfp5-ER was used in all the experiments in our study with the exception of those when the efficiency of the four strains of *A. tumefaciens* was being tested.

Effect of acetosyringone concentration on transient *Agrobacterium*-mediated expression of *gfp* gene in leaves of *N. benthamiana*

There are numerous controversial data on the effect of acetosyringone concentration on transient *gfp* gene expression in *N. benthamiana*. In our study, we monitored the effect of acetosyringone concentration ranging from 50 to 600 μM on the level of GFP fluorescence. Our results indicated that the highest level of GFP fluorescence in the presence of suppressor (HC-Pro), measured as a mean gray value, was reached at 450 μM and remained unchanged up to 600 μM of AS. When agroinfiltration was performed with a control suspension of *Agrobacterium* carrying the binary plasmid pBINmgfp5-ER only a slight in-

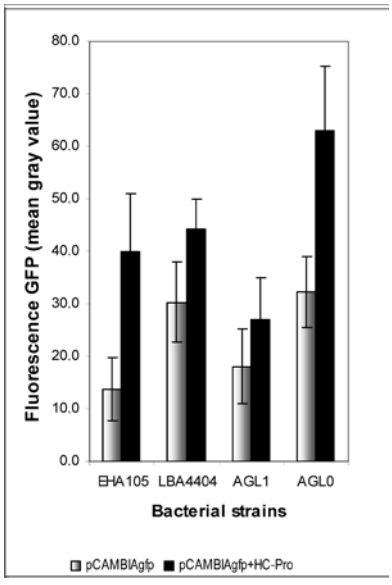

Figure 2. Comparison of the levels transient *gfp* gene expression mediated by four strains *A. tumefaciens* carrying the binary plasmids in the leaves of *N. benthamiana*. The cultures of particular strains (EHA105, AGL0, AGL1 and LBA4404) of *A. tumefaciens* harbouring plasmid pCAMBIA(gfp)1302 were infiltrated singly or in combination with the culture carrying plasmid pJR1Ri-HC-Pro into the leaves of *N. benthamiana* as described in Materials and Methods section. The infiltrated leaves were photographed at 4 dpi. GFP fluorescence was quantified with ImageJ programme. *N. benthamiana* leaves had an average fluorescence as a mean gray value.

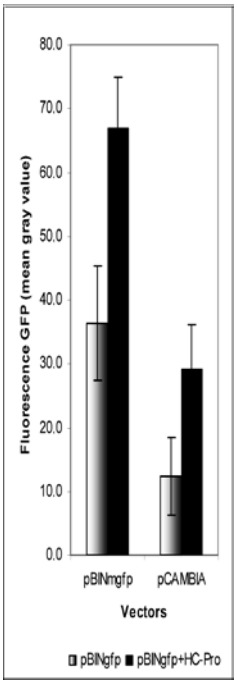

Figure 3. Comparison of the levels transient *gfp* gene expression mediated by strain EHA105 of *Agrobacterium* harbouring binary plasmids pBINmgfp5-ER or pCAMBIA(gfp)1302 in the leaves *N. benthamiana*.
The cultures of EHA105 carrying of the binary plasmids pBINmgfp5-ER, pCAMBIA(gfp)1302 and pJR1Ri-HC-Pro(TuMV) were infiltrated into the leaves of *N. benthamiana* as described in Materials and Methods section. The infiltrated leaves were photographed at 4 dpi. GFP fluorescence was quantified with ImageJ programme.

crease in the *gfp* gene expression was observed when the concentration of acetosyringone was increased from 50 to 600 μM (Fig. 4).

Effect of the density of the *Agrobacterium* suspension on transient *gfp* gene expression

The studies were performed for four different densities of bacterial suspensions (0.1, 0.5, 1.0 and 1.5 OD_{600}). The infiltrated leaves were photographed on the 4th day after agroinfiltration. The results are presented in Fig. 5. We did not notice any differences in the levels of transient *gfp* gene expression for the different densities of *Agrobacterium* suspensions used for infiltration. The co-expression of *gfp* and HC-Pro genes caused an increase in the level of GFP fluorescence but this enhancement was similar for all the densities of bacterial suspension used (the levels of GFP fluorescence with a suppressor were increased twice in relation to that in the absence of suppressor) (Fig. 5). The density of the bacterial suspension used for infiltration during all the experiments reported in this paper was OD_{600} = 1.0.

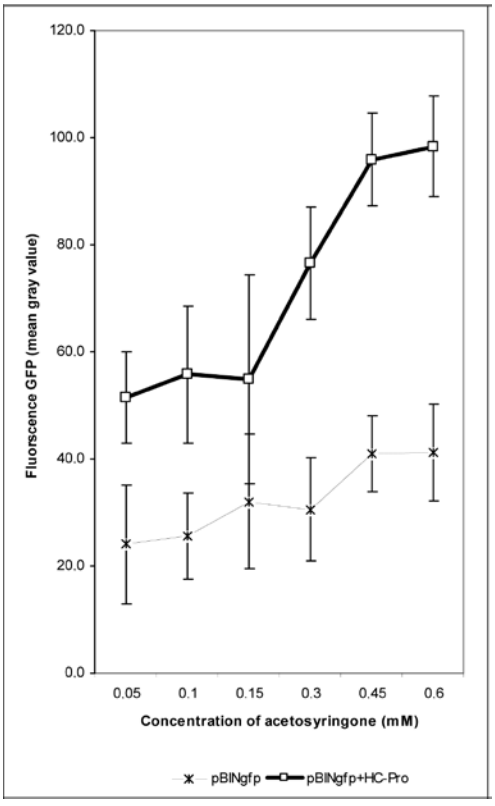

Figure 4. Effect of acetosyringone concentration of on transient expression *gfp* gene in the leaves of *N. benthamiana*.
Leaves of *N. benthamiana* were infiltrated as described in the Materials and Methods section with culture of strain EHA105 harbouring the plasmids pBINmgfp5-ER and pJR1Ri-HC-Pro(TuMV). Photographs were taken at 4 dpi.

Changes of transient *gfp* gene expression levels during days 1–6 after agroinfiltration

In a long term experiment, two cultures of strain EHA105 carrying the binary plasmids pBINmgfp5-ER and pJR1Ri-HC-Pro(TuMV) were infiltrated into leaves of *N. benthamiana*. A combination of two suspensions of *A. tumefaciens* carrying the binary plasmid pBINmgfp5-ER and the empty vector pJR1Ri was infiltrated into the left half-leaves (controls). A second combination of two cultures of *Agrobacterium* carrying the binary plasmids pBINmgfp5-ER and pJR1Ri-HC-Pro(TuMV) was infiltrated into the right half-leaves. The infiltrated leaves were photographed on each day from the 1st until the 6th day after agroinfiltration. The GFP fluorescence intensity on days 1–6 after agroinfiltration was measured at three random selected sites in non-infiltrated zone (as a background) and three sites in the agroinfiltrated zone. Six data sets were collected over the time period of 6 days (Fig. 6). The GFP fluorescence on the left half-leaves (infiltrated with *Agrobacterium* carrying the binary plasmids pBINmgfp5-ER and pJR1Ri) reached about 30 as the mean gray value at day 4 then remained unchanged until day 6. The GFP fluorescence of the *N. benthamiana* leaves agroinfiltrated with the suspension of strain EHA105 of *Agrobacterium* carrying the binary plasmids pBINmgfp5-ER and pJR1Ri-HC-Pro at first appeared on the second day post-agroinfiltration and then increased until the end of the experiment on the sixth day (GFP fluorescence was at least about

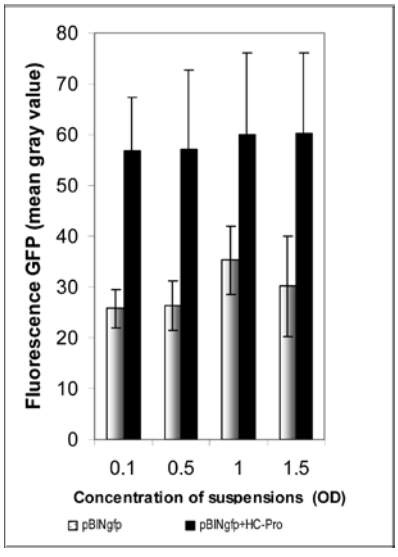

Figure 5. Effect of density of *Agrobacterium* suspension on transient *gfp* gene expression.
The four different densities of *Agrobacterium* suspension: 0.1, 0.5, 1.0 and 1.5 OD at 600 nm carrying of the binary plasmids pBINmgfp5-ER and pJR1Ri-HC-Pro(TuMV) were infiltrated into the leaves of *N. benthamiana* as described in the Materials and Methods section. The infiltrated leaves were photographed at 4 dpi.

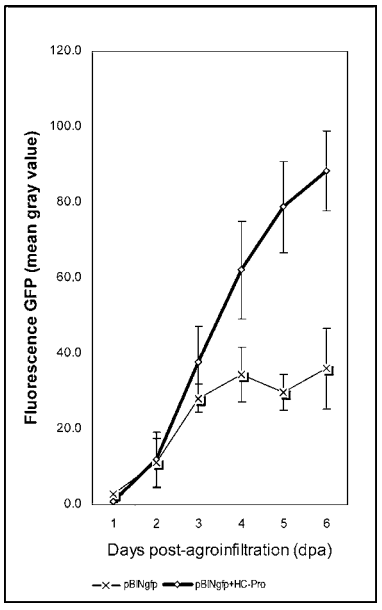

Figure 6. **Course of transient *gfp* gene expression within 1–6 days post-agroinfiltration**.
The two cultures of strain EHA105 carrying of the binary plasmids pBINmgfp5-ER and pJR1Ri-HC-Pro(TuMV) were infiltrated into the leaves of *N. benthamiana* plants as described in the Materials and Methods section. The infiltrated leaves were photographed on each day from the 1st until the 6th day post-agroinfiltration.

3-fold higher than that in the leaves infiltrated with pBINmgfp5-ER in the absence of HC-Pro).

Effect of leaf ageing on transient *gfp* gene expression in leaves of *N. benthamiana*

For comparative purposes, leaves from three different positions corresponding to different leaf age were selected for agroinfiltration: the top leaf position (youngest leaves), intermediate leaf position and bottom leaf position (oldest leaves) (Fig. 7A). A culture of *A. tumefaciens* carrying pBINmgfp5-ER and the empty vector-pJR1Ri was infiltrated into the left half-leaves, and the right half-leaves of these plants were infiltrated with a suspension of *Agrobacterium* carrying the binary plasmids pBINmgfp5-ER and pJR1Ri-HC-Pro(TuMV) at the same time. The inoculated leaves were photographed on the 4th day after agroinfiltration. In the leaves agroinfiltrated with the control suspension, the GFP fluorescence intensity was similar at all leaf positions with the mean gray value ranging from 17 to 24 (Fig. 7B). The co-infiltration with suspension carrying the *gfp* and HC-Pro genes resulted in strong expression of the *gfp* gene in the youngest leaves (the top position) and the GFP fluorescence intensity was three times higher in relation to that of the leaves infiltrated with pBINmgfp5-ER plasmid without HC-Pro. A similar increase was observed in the intermediate position leaves but the levels of GFP fluorescence in the presence and without the suppressor were slightly lower. At the

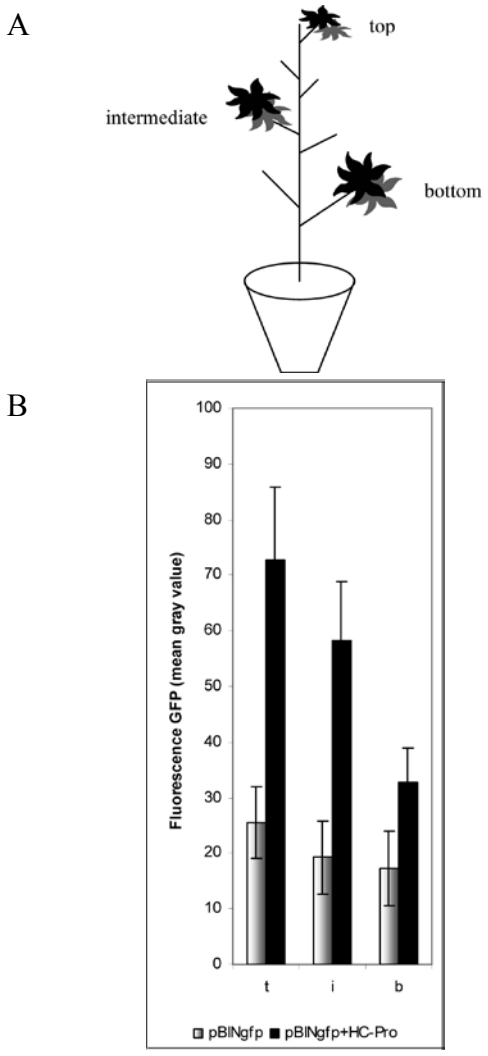

Figure 7. **(A) Three different leaf positions corresponding to different leaf age were selected for agroinfiltration**. **(B) Effect of leaf ageing on transient *gfp* gene expression in the leaves of *N. benthamiana*.**
The combination two cultures of *A. tumefaciens* carrying of the binary plasmids pBINmgfp5-ER and pJR1Ri-HC-Pro(TuMV) was infiltrated into the leaves of *N. benthamiana* as described in the Materials and Methods section. The infiltrated leaves were photographed at 4 dpi.

bottom position (oldest leaves) of *N. benthamiana* the level of GFP fluorescence with the HC-Pro gene was increased twice in relation to that without the HC-Pro gene (Fig. 7B). The GFP fluorescence of *N. benthamiana* leaves expressed as a mean gray value varied from 11 to 23 in the leaves infiltrated with *gfp* without the HC-Pro gene, and from 28 to 38 in the leaves infiltrated with the *gfp* and HC-Pro genes.

Effect of the three viral suppressors on transient *Agrobacterium*-mediated *gfp* gene expression in leaves of *N. benthamiana*

For comparative purposes, the three suppressors (HC-Pro-TuMV, HC-Pro-PVY and P1/HC-Pro TuMV) were used during transient expression in

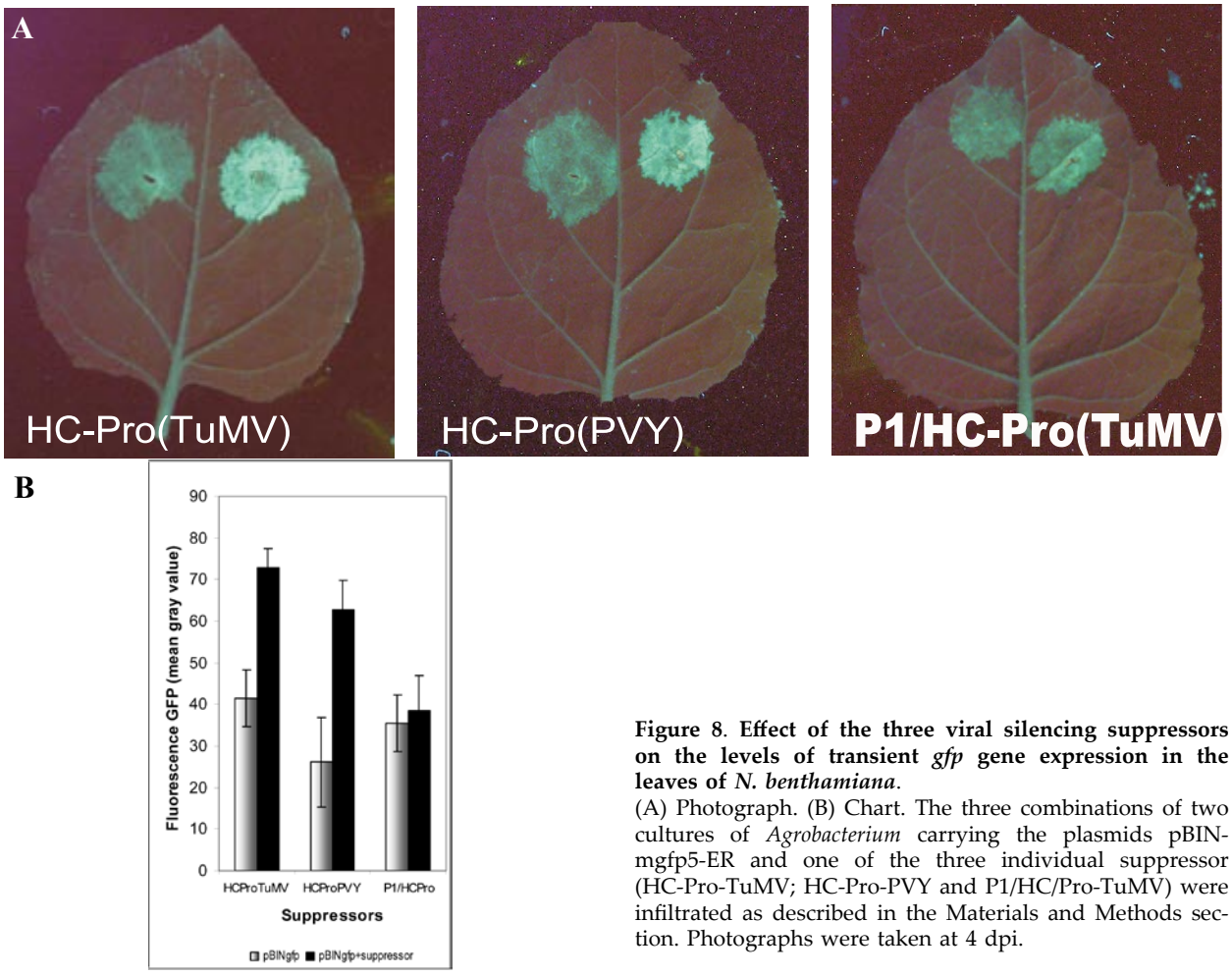

Figure 8. Effect of the three viral silencing suppressors on the levels of transient *gfp* gene expression in the leaves of *N. benthamiana*.
(A) Photograph. (B) Chart. The three combinations of two cultures of *Agrobacterium* carrying the plasmids pBINmgfp5-ER and one of the three individual suppressor (HC-Pro-TuMV; HC-Pro-PVY and P1/HC/Pro-TuMV) were infiltrated as described in the Materials and Methods section. Photographs were taken at 4 dpi.

leaves of *N. benthamiana*. Suspension of *A. tumefaciens* carrying the binary plasmids: pBINmgfp5-ER and empty vector pJR1Ri was infiltrated into the left half-leaves (controls). Three cultures of *Agrobacterium* carrying the binary plasmid pBINmgfp5-ER and one of the three viral silencing suppressors in the binary plasmid (HC-Pro-TuMV; HC-Pro-PVY and P1/HC/Pro-TuMV) were infiltrated into the right half-leaves. The cultures of *Agrobacterium* at an optical density 1.0 carrying the binary plasmids were mixed at the 1:1 ratio. The infiltrated leaves were photographed on the 4th day after agroinfiltration. The data presented in Figs 8A and 8B enabled us to draw a conclusion that both suppressors HC-Pro-TuMV and HC-Pro-PVY are equally efficient since they both resulted in the same level of increase in GFP fluorescence over that present in the absence of viral suppressors. When P1/HC-Pro proteins from TuMV were used during agroinfiltration, no suppression of silencing was observed.

DISCUSSION

Voinnet *et al.* (2003) published a protocol for the transient transformation of *N. benthamiana* based-on a co-expression of viral suppressors of gene silencing. Here we report a further optimization of this system by the application of the strains EHA105 and AGL0 of *Agrobacterium tumefaciens* in addition to increased concentration of acetosyringone during bacterial preincubation.

The green fluorescent protein (*gfp*) gene was used as a sensitive and non-invasive marker to visualize transient gene expression in transformed cells. When the *gfp* gene alone was used for transient transformation, the maximum level of expression was rather low. It was much enhanced when a mixture of the *gfp* and HC-Pro genes at the 1:1 ratio was used for transient transformation. The results of our study provide circumstantial evidence that the transient *gfp* gene expression was restricted by local RNA silencing and that this silencing was suppressed by viral proteins (HC-Pro) from TuMV or PVY viruses, being well-known suppressors of RNA silencing (Kasschau & Carrington, 1998; Anandalakshmi *et al.*, 1998).

Our data reveal that plasmid pBINmgfp5-ER is more efficient for transient *gfp* gene expression in leaves of *N. benthamiana* than pCAMBIA(gfp)1302. Different levels of green fluorescence observed after agroinfiltration with each of the plasmids could

be explained by differences between the variants of the *gfp* gene contained in those plasmids. Plasmid pCAMBIA1302 contains the *mgfp5* version of *gfp* (Siemering *et al.,* 1996) whereas the *gfp* gene contained in plasmid pBINmgfp5-ER is a version further modified by the addition of a signal sequence which targets mGFP5-ER to the endoplasmic reticulum (Haseloff *et al.,* 1997).

The analysis of the *gfp* gene expression level measured as the intensity of GFP fluorescence without a suppressor (HC-Pro) showed that this expression was increasing from days 2 to 4 then it remained unchanged up to day 6. In contrast, when this analysis was performed in the presence of the suppressor (HC-Pro-TuMV) the GFP fluorescence was increasing from the 1st day until the end of the assay (6th day). Our results support the hypothesis put forward by Johansen and Carrington (2001) and Voinnet *et al.* (2003) that after the transient transformation local RNA silencing is triggered and then suppressed in the presence of some viral suppressor.

Figure 7B shows the GFP fluorescence in *N. benthamiana* leaves of different ages. The highest GFP fluorescence was found at the top leaf position (youngest leaves), at intermediate and bottom positions it was lower (older leaves). This was true both in the presence and in the absence of a suppressor. Halfhill *et al.* (2003) suggested that changes in GFP fluorescence were related to changes in the concentration of soluble proteins during leaf ageing. The level of *gfp* gene expression and concentration of soluble proteins declined at similar times and to similar extents in individual leaves at different positions. The close relationship between these two factors would suggest that the GFP decline was a result of general changes in leaf physiology and not a specific effect on the transgene or its expression controlled by the 35S promoter.

Acetosyringone (AS) is known to induce the *vir* operon of *A. tumefaciens* (Hiei *et al.,* 1994). We also monitored the effect of concentration of acetosyringone during incubation of *Agrobacterium* cultures. The highest level of GFP fluorescence in the presence of a suppressor was reached at 450 μM and remained unchanged up to 600 μM AS. An increase in the concentration of acetosyringone from 50 to 600 μM resulted in a slight increase in the *gfp* gene expression (GFP fluorescence) when the agroinfiltration was performed without a suppressor. Jeoung *et al.* (2002) have reported optimization of acetosyringone concentration in the preculture and co-culture medium for transient expression of *gfp* gene in three inbred lines of sorghum at four different concentrations of AS ranging from 200 to 1000 μM. The concentration of AS in culture medium was found to affect the transient gene expression. Further, the optimum AS concentration varied for different inbred lines.

Although Voinnet *et al.* (2003) showed that the p19 protein of tomato bushy stunt virus (TBSV) was an effective suppressor of silencing we decided to use the HC-Pro proteins of turnip mosaic virus (TuMV) and potato virus Y (PVY) in our studies as these proteins are the main objects of our interest. The data presented in this report supports the earlier observations on HC-Pro-TuMV and HC-Pro-PVY (Kasschau & Carrington, 1998; Anandalakshmi *et al.,* 1998). Here they were equally effective since they both resulted in the same level of increase in GFP fluorescence over that detected in the absence of viral suppressors. However, unexpectedly the proteins P1/HC-Pro(TuMV) did not result in an increase in GFP fluorescence. This discrepancy may be explained by the fact that the proteins HC-Pro-TuMV and HC-Pro-PVY encoded in the constructs pJR1Ri-HC-Pro-TuMV and pJR1Ri-HC-Pro-PVY had methionine at the N terminus as a result of addition of the initiation codon ATG to the 5′ termini of their genes during construct preparation. When two genes P1 and HC-Pro were used in the construct the initiation codon was at the 5′-terminus of P1 and it was not added to the beginning of HC-Pro, which can imply differences in the three-dimensional structure of the HC-Pro protein in both constructs. In contrast, Pruss *et al.* (1997) suggested that protein P1 might enhance HC-Pro activity as a suppressor of RNA silencing.

In general, we confirmed that the *gfp* gene can be used effectively as a reporter for optimizing the conditions of successful expression during transient *Agrobacterium*-mediated transformation of leaves of *N. benthamiana.* The results obtained indicate that the transient *gfp* gene expression was related to leaf aging (the maximum expression was observed in the youngest leaves), depended on acetosyringone concentration during incubation of *Agrobacterium* (maximum *gfp* gene expression at 450–600 μM AS) and was independent of the density of the bacterial suspension. Much higher levels of transient expression were observed when the strains AGL0 and EHA105 of *A. tumefaciens* were used compared to AGL1 and LBA4404. The HC-Pro proteins from TuMV and PVY viruses were equally effective since they both resulted in the same level of increase in GFP fluorescence over that present without of viral suppressors.

Voinnet *et al.* (2003) have demonstrated the use of transient expression in the presence of suppressors in another plant and for other viral genes expressed either individually or in mixtures (*Cf-4*, *Cf-9* and *NIa*). Vaquero *et al.* (1999) have described the transient expression of different forms of a tumor-specific chimeric antibody (rAbs) in tobacco leaves. The production of full-size human chimeric antibody was achieved by simultaneous expression

of the heavy and light chains. The genes were transferred into tobacco leaf cells by agroinfiltration in two independent *Agrobacterium* cultures. This result demonstrates that multimeric proteins can be analyzed by using *Agrobacterium*-mediated transient expression. Several genes encoding proteins can be simultaneously transiently expressed within the same cells. This enables analysis of complex multimeric proteins. In stable transgenic plants such manipulation would require several independent transformations that could take months.

The improvements of the *Agrobacterium*-mediated transient expression system reported here will be important in the identification of new viral suppressors of RNA silencing, for systematic investigation of unidentified genes and also for industrial production of proteins in plants. In our laboratory, this system will be used also to test expression of constructs before generation of stably transformed plants.

Acknowledgements

We would like to thank Joanne L. Elson for critical reading of the manuscript. We thank also Jacek Augustyniak and Andrew Greenland for kindly providing the vectors. We thank Paweł Piwko for help in the initial experiments on agroinfiltration of *Nicotiana benthamiana* leaves and for assistance in photographing the leaves.

REFERENCES

Al-Kaff NS, Covey SN, Kreike MM, Page AM, Pinder R, Dale PJ (1998) Transcriptional and posttranscriptional plant gene silencing in response to a pathogen. *Science* **279**: 2113–2115.

Anandalakshmi R, Pruss GJ, Ge X, Marathe R, Mallory AC, Smith TH, Vance VB (1998) A viral suppressor of gene silencing in plants. *Proc Natl Acad Sci USA* **95**: 13079–13084.

Baulcombe DC (1996) RNA as a target and an initiator of post-transcriptional gene silencing in transgenic plants. *Plant Mol Biol* **32**: 79–88.

Baulcombe D (2004) RNA silencing in plants. *Nature* **431**: 356–363.

Baulcombe DC, Malnár A (2004) Crystal structure of p19 — a universal suppressor of RNA silencing. *Trends Biochem Sci* **29**: 279–281.

Dougherty WG, Parks TD (1995) Transgenes and gene suppression: telling us something new? *Curr Opin Cell Biol* **7**: 399–405.

English JJ, Mueller E, Baulcombe DC (1996) Suppression of virus accumulation in transgenic plants exhibiting silencing of nuclear genes. *Plant Cell* **8**: 179–188.

Fischer R, Vaquero-Martin C, Sack M, Drossard J, Emans N, Commandeur U (1999) Towards molecular farming in the future: transient protein expression in plants. *Biotechnol Appl Biochem* **30**: 113–116.

Fischer R, Stoger E, Schillberg S, Christou P, Twyman R (2004) Plant-based production of biopharmaceuticals. *Curr Opin Plant Biol* **7**: 152–158.

Halfhill MD, Millwood RJ, Rufty TW, Weissinger AK, Stewart CN (2003) Spatial and temporal patterns of green fluorescent protein (GFP) fluorescence during leaf canopy development in transgenic oilseed rape, *Brassica napus* L. *Plant Cell Rep* **22**: 338–343.

Hamilton A, Voinnet O, Chappell L, Baulcombe D (2002) Two classes of short interfering RNA in RNA silencing. *EMBO J* **21**: 4671–4679.

Haseloff J, Siemering KR, Prasher DC, Hodge S (1997) Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. *Proc Natl Acad Sci USA* **94**: 2122–2127.

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J* **6**: 271–282.

Horn ME, Woodard SL, Howard JA (2004) Plant molecular farming: systems and products. *Plant Cell Rep* **22**: 711–720.

Jeoung JM, Krishnaveni S, Muthukrishnan S, Trick HN, Liang GH (2002) Optimization of sorghum transformation parameters using genes for green fluorescent protein and β-glucuronidase as visual markers. *Hereditas* **137**: 20–28.

Johansen LK, Carrington JC (2001) Silencing on the spot. Induction and suppression of RNA silencing in the *Agrobacterium*-mediated transient expression system. *Plant Physiol* **126**: 930–938.

Kapila J, DeRycke R, VanMontagu M, Angenon G (1997) An *Agrobacterium*-mediated transient gene expression system for intact leaves. *Plant Sci* **122**: 101–108.

Kasschau KD, Carrington JC (1998) A counterdefensive strategy of plant viruses: suppression of posttranscriptional gene silencing. *Cell* **95**: 461–470.

Kozak M (1984) Point mutations close to the AUG initiator codons affect the efficiency of translation of rat preproinsulin *in vivo*. *Nature* **308**: 241–246.

Lakatos L, Szittya G, Silhavy D, Burgyan J (2004) Molecular mechanism of RNA silencing suppression mediated by p19 protein of tombusviruses. *EMBO J* **23**: 876–884.

Lindbo JA, Silva-Rosales L, Proebsting WM, Dougherty WG (1993) Induction of a highly specific antiviral state in transgenic plants: implications for regulation of gene expression and virus resistance. *Plant Cell* **5**: 1749–1759.

Mallory AC, Reinhart BJ, Bartel D, Vance VB, Bowman LH (2002) From the cover: a viral suppressor of RNA silencing differentially regulates the accumulation of short interfering RNAs and mikro-RNAs in tobacco. *Proc Natl Acad Sci USA* **99**: 15228–15233.

Maximova SN, Dandekar AM, Guiltinan MJ (1998) Investigation of *Agrobacterium*-mediated transformation of apple using green fluorescent protein: high transient expression and low stable transformation suggest that factors other than T-DNA transfer are rate-limiting. *Plant Mol Biol* **37**: 549–559.

Pruss G, Ge X, Shi XM, Carrington JC, Vance VB (1997) Plant viral synergism: the potyviral genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses. *Plant Cell* **9**: 858–868.

Qu F, Ren T, Morris TJ (2003) The coat protein of turnip crincle virus suppresses posttranscriptional gene silencing at an early initiation step. *J Virol* **77**: 511–522.

Reed JC, Kasschau KD, Prokhnevsky AI, Gopinath K, Pogue GP, Carrington JC, Dolja VV (2003) Suppressor of RNA silencing encoded by *Beet yellows virus*. *Virology* **306**: 203–209.

Roth BM, Pruss GJ, Vance VB (2004) Plant viral suppressors of RNA silencing. *Virus Res* **102**: 97–108.

Sambrook J, Russell DW (2001) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Siemering KR, Golbik R, Sever R, Haseloff J (1996) Mutations that suppress the thermosensitivity of green fluorescent protein. *Curr Biol* **6**: 1653–1663.

Smith HA, Swaney SL, Parks TD, Wernsman EA, Dougherty WG (1994) Transgenic plant virus resistance mediated by untranslatable sense RNAs: expression, regulation, and fate of nonessential RNAs. *Plant Cell* **6**: 1441–1453.

Tenllado F, Llave C, Diaz-Ruiz JR (2004) RNA interference as a new biotechnological tool for the control of virus diseases in pants. *Virus Res* **102**: 85–96.

Thomas CL, Leh V, Lederer C, Maule AJ (2003) Turnip crincle virus coat protein mediates suppression of RNA silencing in *Nicotiana benthamiana*. *Virology* **306**: 33–41.

Vaquero C, Sack M, Chandler J, Drossard J, Schuster F, Monecke M, Schillberg S, Fischer R (1999) Transient expression of a tumor-specific single-chain fragment and a chimeric antibody in tobacco leaves. *Proc Natl Acad Sci USA* **96**: 11128–11133.

Voinnet O, Lederer C, Baulcombe DC (2000) A viral movement protein prevents systemic spread of the gene silencing signal in *Nicotiana benthamiana*. *Cell* **103**: 157–167.

Voinnet O, Rivas S, Mestre P, Baulcombe D (2003) An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *Plant J* **33**: 949–956.