pCB301-p19: A Binary Plasmid Vector to Enhance Transient Expression of Transgenes by Agroinfiltration

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Introduction

The p19 protein is encoded by one of the two nested genes (p19 and p22) of the tomato bushy stunt virus (TBSV) and is thought to be involved in symptom determination of TBSV (Scholthof *et al*., 1995). It was later discovered that the p19 protein functions as a suppressor of post-transcriptional gene silencing (PTGS) in *Nicotiana benthamiana* (Voinnet *et al*., 1999). Voinnet *et al* (1999) showed that, in a GFP-transgenic *N. benthamiana* plants, p19 was able to revert the gene silencing caused by infection with *Agrobacterium tumefaciens* carrying a GFP construct (pBin19-35S:GFP). A useful application for the p19 protein was put forward by Voinnet *at al* (2003). The authors showed that they were able to enhance the over-expression of transgenes in *N. benthamiana* by including agrobacteria carrying the p19 constructs along with the binary vectors carrying the other gene constructs in their agroinfiltration experiments (Voinnet *et al*., 2003). The p19 protein was also shown to be the most effective in increasing the transient expression of a transgene in *N. benthamiana* when it was compared to the enhancements achieved by other PTGS suppressors including HcPro (potato virus Y) and p25 (potato virus X) (Voinnet *et al*., 2003). In addition, p19 was able to enhance the expression of several diverse proteins including GFP (delivered as 35S:GFP), Nla protease (35S:Nla:HA), CF9 (35S:Cf-9:myc, 35S:Cf9-TAP) and CF4 (35S:Cf-4:myc, 35S:Cf4-TAP). The yield gain due to p19 was reported to be in excess of 50 folds (in case of Nla protease) compared to the expression level without the p19 protein, and 37 folds in case of GFP.

The plasmid pCB301-I1M (Fig. 1, top, constructed by Dr. Walid Hamada, see Hamada and Kamoun, http:www.KamounLab.net) is a binary vector derived from mini-binary vector pCB301 (made by deleting 5kb non-T-DNA sequence from pBIN19) (Xiang *et al*., 1999) and contains the PR1:Inf1 ORF (Kamoun *et al*., 1999) in an expression cassette consisting a 35S promoter, a TMV omega leader sequence (translation enhancer) and a potato proteinase inhibitor-II polyadenylation region (terminator TPI-II). The PR1:Inf1 ORF can be replaced by a gene of interest by using *Nco*I and *Sac*I restriction sites. The pCB301-I1M plasmid is useful for transiently expressing the inserted gene by agroinfiltration. This report describes the cloning of p19 ORF into the pCB301-I1M by replacing the PR1:Inf1-M ORF.

Cloning strategy

The p19 ORF sequence did not contain *Nco*I and *Sac*I sites. Therefore, it can be cloned into pCB301-I1M using these sites (Fig. 1).

A pair of PCR primers was designed from the p19 ORF in TBSV (Kindly donated by Dr Herman Scholthof, Texas A&M University, Texas). The forward primer contained the *Nco*I site and reverse primer contained the *Sac*I site (p19-NcoIF – 5'CGAACAAGTCAATAAACCATGG3' and p19-SacIR – 5'CCGGAGCTCAGA GTCTGTCTTACTCGCCTTCT3', respectively. Oligo box 8, -80° C freezer, Kamoun's lab). The p19 ORF was amplified by Pfu-PCR using the TBSV genome as a template. The PCR products were cleaned with Qiagen PCR purification kit and digested with *Nco*I and *Sac*I. The same enzymes were used to digest the pCB301-I1M plasmid DNA. Digested PCR products and the vector fragments were gel-purified using the Qiagen gel extraction kit and ligated with T4 DNA ligase. *A. tumefaciens* GV3101 cells were transformed with the ligation reaction and the transformants were selected on LB+Kan (50 µg/ml) agar plates. The transformants were screened by colony-PCR for presence of the inserts using the above primer pairs. Three colonies that gave the PCR products with appropriate sizes were selected for sequencing of the insert. For sequencing, the whole expression cassette of the inserts were amplified using a primer pair designed from 5' end of 35S and the 3' end of TPII sequence (35S-NR – 5'GTCAACATGGTGGAGCACG

A3' and TPII-NR – 5'GGTTAATAAAATGCATCTGG3'). PCR products were gel-purified and sequences (for both strands) were obtained using the 35S-NR and TPII-NR primers.

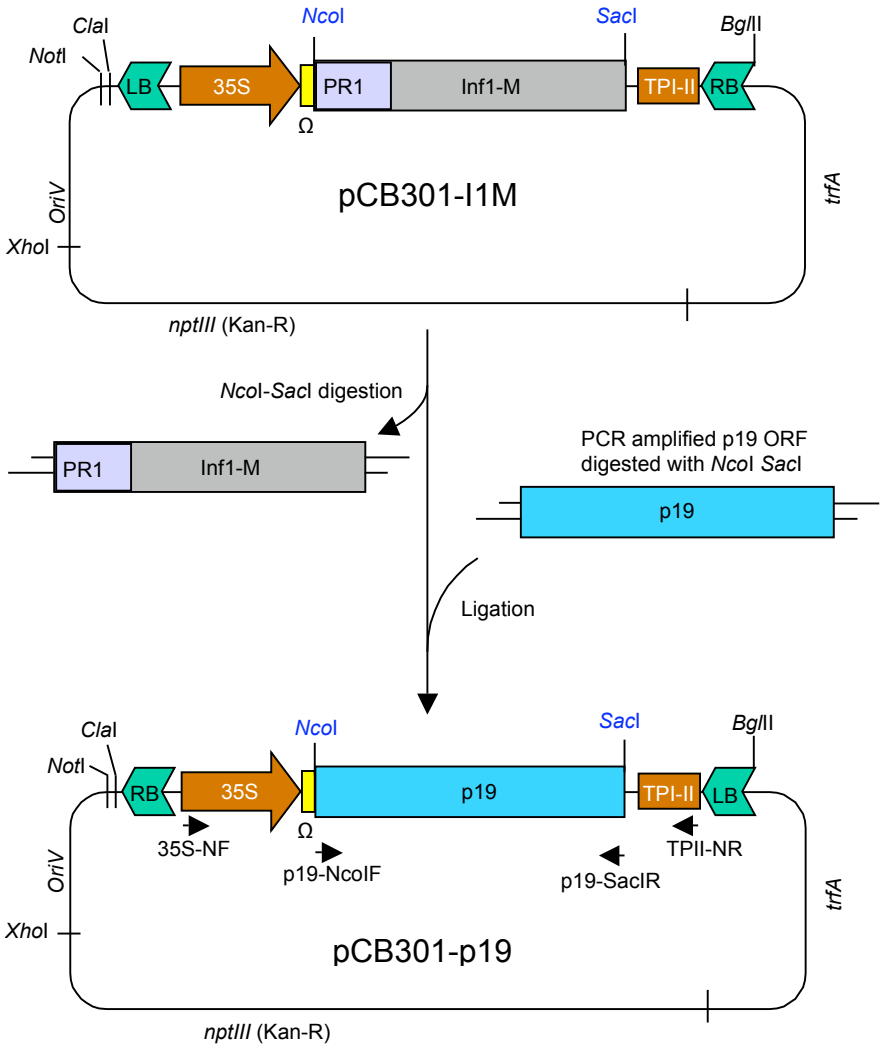

Fig. 1. Cloning strategy to replace PR1:Inf1-M with the p19 ORF in pCB302-I1M binary cloning vector. (Note: The figures are not drawn to scale).

Sequence reads from three clones were checked for p19 ORF and cloning site integrity for both ends of the ORF. All three clones had identical sequences. Clone #2 was further sequenced using p19-NcoIF and p19-SacIR primers. The sequence-checking confirmed that cloning sites were recreated properly and there were no nucleotide substitution/mutation in the p19 ORF (Fig. 2). The 35S promoter region (30-422 bp) showed 100% identity to the 35S promoters of several binary vectors from GenBank (eg. acc. # AY456904), the omega leader (423-492 bp) showed 100% identity to other omega-prime leader sequences (eg. acc. # AY183361), the p19 ORF (493-1011bp) showed 100% identity to tomato bushy stunt mRNA for p19 and p22 (acc. # Z68901), and the terminator TPI-II region (1034-1254) showed 100% identity to the potato proteinase inhibitor gene (acc. # X04118).

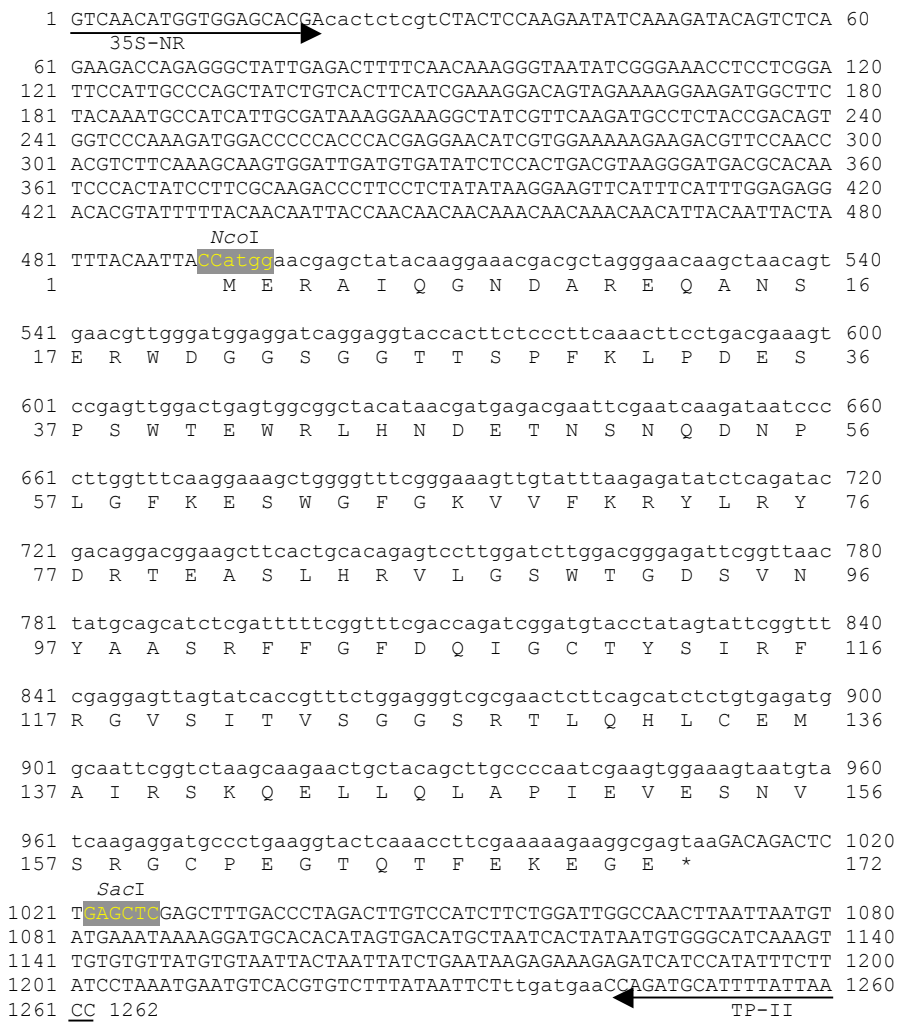

Fig. 2. Consensus nucleotide sequence derived from the sequence reads of pCB301-p19 clone #2. The primer sequences used to amplify the fragment are underlined with arrows. *Nco*I and *Sac*I cloning sites are shaded in grey. The p19 ORF is shown in lower-case letters between the cloning sites with the translated amino acid sequences shown below the ORF. The sequences shown in lower-case letters near the primers could not be called clearly due to very strong signals in chromatograms at the start of each sequencing reads.

Testing of pCB301-p19 construct using a GFP reporter construct

The following constructs were used together with the p19 construct to ascertain the p19-enhanced transient expression of a transgene in *N. benthamiana* by agroinfiltration.

1. 35S-GFP A pBin19-based vector from Voinnet *et al* (2003) for transient expression of GFP. This construct is in *Agrobacterium* C58-C1
2. pCB302-3 An empty vector used as negative control for background fluorescence (in *Agrobacterium* GV3101)
3. pCB301-p19 In *Agrobacterium* GV3101.

Experimental procedure:

Agrobacteria (from single colonies) containing the constructs were grown in 15 ml LB+Kan (50 □g ml^{-1}) broth until OD_{600} reached ~ 0.5-0.7. Cells were pelleted by centrifugation at 5000g for 15 min. The pellets were resuspended in induction buffer (10 mM MES pH5.6, 10 mM $MgCl_2$, 150 □M acetosyringone) adjusting the OD_{600} to 0.7 and incubated for 2 h. Agrobacteria suspensions were mixed as below (where applicable) and infiltrated into *N. benthamiana* leaves.

(a) un-infiltrated control leaves
(b) pCB302-3 (undiluted)
(c) 35S-GFP + pCB302-3 (5 ml + 5 ml)
(d) 35S-GFP + pCB301-p19 (5 ml + 5 ml)

Infiltrated leaves were observed under UV light 5 days post infiltration using a dissecting microscope attached with a digital camera and a GFP filter. Exposure time was 35s for each leaf.

Results and conclusions

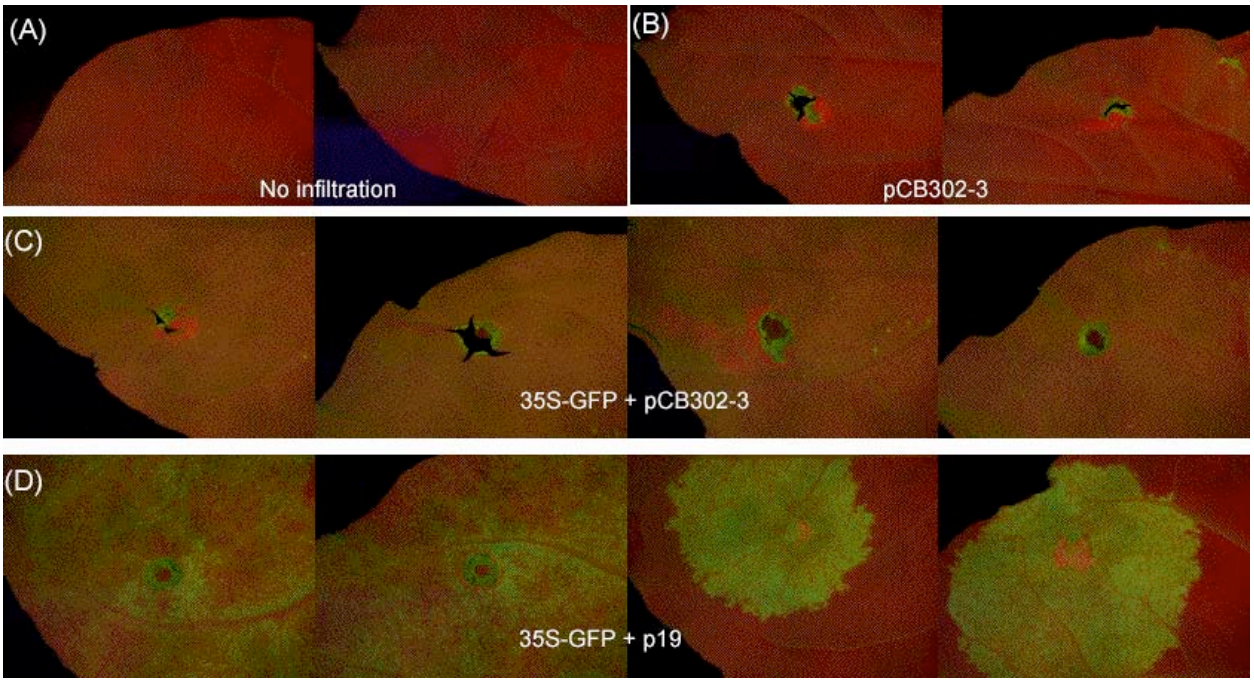

Fig. 3. Leaves of *N. benthamiana* infiltrated with various gene constructs observed under UV light using a GFP filter. (A) Leaves with no infiltration. Leaves infiltrated with (B) pCB302-3 only, (C) 35S-GFP construct, and (D) 35S-GFP + pCB301-p19

The GFP fluorescence was consistently stronger in infiltrations that included the p19 construct (Fig. 3, panel D) than the infiltrations without it. The p19 construct was able to enhance the expression of 35S:GFP construct as reported by Voinnet et al. (2003). The amounts of GFP fluorescence were not measured, so the magnitude of enhancement by p19 could not be determined. This pCB301-p19 construct was deposited in “Agrobacterium plasmid Stock Box 3” in Kamoun’s lab.

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TECHNICAL ADVANCE

An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus

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Summary

Transient gene expression is a fast, flexible and reproducible approach to high-level expression of useful proteins. In plants, recombinant strains of *Agrobacterium tumefaciens* can be used for transient expression of genes that have been inserted into the T-DNA region of the bacterial Ti plasmid. A bacterial culture is vacuum-infiltrated into leaves, and upon T-DNA transfer, there is ectopic expression of the gene of interest in the plant cells. However, the utility of the system is limited because the ectopic protein expression ceases after 2–3 days. Here, we show that post-transcriptional gene silencing (PTGS) is a major cause for this lack of efficiency. We describe a system based on co-expression of a viral-encoded suppressor of gene silencing, the p19 protein of tomato bushy stunt virus (TBSV), that prevents the onset of PTGS in the infiltrated tissues and allows high level of transient expression. Expression of a range of proteins was enhanced 50-folds or more in the presence of p19 so that protein purification could be achieved from as little as 100 mg of infiltrated leaf material. The effect of p19 was not saturated in cells that had received up to four individual T-DNAs and persisted until leaf senescence. Because of its simplicity and rapidity, we anticipate that the p19-enhanced expression system will have value in industrial production as well as a research tool for isolation and biochemical characterisation of a broad range of proteins without the need for the time-consuming regeneration of stably transformed plants.

Keywords: *Agrobacterium*-mediated transient expression, RNA silencing, viral suppressors, p19 protein, protein purification.

Introduction

The use of plants as expression systems for valuable recombinant proteins often involves integration of a transgene into the plant genome (Giddings *et al.*, 2000). However, transient expression systems are also useful because they are fast, flexible, unaffected by chromosomal positional effects and can be used in fully differentiated plant tissues (Fischer *et al.*, 1999). For example, virus vectors allow expression of foreign genes at higher levels in infected tissues than is normally the case in transformed plants (Porta *et al.*, 1996; Yusibov *et al.*, 1999). *Agrobacterium tumefaciens* can also be used in transient expression (Fischer *et al.*, 1999). *Agrobacterium*, infiltrated into plant leaves as a liquid culture, mediates transfer of transgenes from the T-DNA region of the bacterial Ti plasmid molecules into the plant cells (Kapila *et al.*, 1997). Most of the plant cells in the infiltrated region express the transgene (Kapila *et al.*, 1997).

The *Agrobacterium* system, unlike viral vectors, does not lead to systemic expression of the foreign gene. However, it can be used with long (>2 kb) genes that are genetically unstable in virus vectors (Porta *et al.*, 1996). A further advantage of the *Agrobacterium* system is the facility to deliver several transgenes into the same cells (Kapila *et al.*, 1997) so that multimeric proteins, such as antibodies, can be expressed and assembled (Vaquero *et al.*, 1999). The transgenes to be co-expressed are present in different *Agrobacterium* cultures that are mixed prior to infiltration.

In principle, the *Agrobacterium* infiltration system can be applied on an industrial scale. However, the level of transgene expression usually peaks at 60–72 h post-infiltration and declines rapidly thereafter. Originally, it was thought that the expression was transient because the bacterial strain/host plant combination was inappropriate and T-DNA transfer was suboptimal. More recently, post-transcriptional gene silencing (PTGS) was proposed as another limiting factor (Johansen and Carrington, 2001).

Post-transcriptional gene silencing is a nucleotide sequence-specific RNA turnover mechanism that is highly conserved among most, if not all, eukaryotes (Hammond *et al.*, 2001). Common features of PTGS in different organisms are the involvement of double-stranded (ds)RNA as initiator molecule (Fire *et al.*, 1998; Hammond *et al.*, 2001) and the presence of short-interfering (si)RNAs of 21–25nt that are processed from dsRNA by an RNAase III-like enzyme (Bernstein *et al.*, 2001; Elbashir *et al.*, 2001; Hamilton and Baulcombe, 1999). The siRNAs confer sequence specificity to a nuclease that degrades any RNA-sharing sequence homology to the activating dsRNA molecules (Hammond *et al.*, 2000). In plants, PTGS operates as an adaptive immune system targeted against viruses (Ratcliff *et al.*, 1999; Voinnet, 2001), and as a counter-defensive strategy, many plant viruses have evolved proteins that suppress various steps of the mechanism (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau and Carrington, 1998; Voinnet, 2001; Voinnet *et al.*, 1999).

Here, we show that PTGS is a general plant response that limits the efficiency of *Agrobacterium*-mediated transient expression. We further demonstrate that transient co-expression of viral suppressors of PTGS alleviates the host silencing response in wild-type *Nicotiana benthamiana*. The most effective suppressor was the p19 protein encoded by tomato bushy stunt virus (TBSV). This protein dramatically enhanced transient expression of a broad range of proteins, allowing, in several instances, to yield gains that were in excess of 50-folds. This system was used to purify a soluble protein by immunoaffinity from as little as 100 mg of infiltrated leaf material. Moreover, the effect of p19 was not saturated in cells that had received up to four individual T-DNA constructs and was manifested until leaf senescence. These findings indicate that transient expression in the presence of suppressors of silencing may have value in industrial production and as a research tool for isolation and functional characterisation of proteins.

Results

Previously, we have shown that PTGS of a stably integrated and highly expressed GFP transgene (35S:GFP; Figure 1) in *N. benthamiana* can be initiated by leaf infiltration with an *Agrobacterium* culture carrying the same GFP construct (Voinnet *et al.*, 1998; Figure 1). PTGS was manifested in the *Agrobacterium*-infiltrated area as a progressive loss of green fluorescence and GFP mRNAs (Voinnet *et al.*, 1998). If the 35S:GFP initiator of silencing was expressed together with a virus-encoded suppressor of PTGS, there was no silencing of GFP (Voinnet *et al.*, 2000). Thus, in leaves that were co-infiltrated with two strains of *Agrobacterium* carrying transgenes for the 35S:GFP initiator and for the PVX-encoded p25 suppressor (35S:p25; Figures 1 and 2a), the GFP fluorescence was brighter than in similar tissues that had been co-infiltrated with 35S:GFP and water (Figure 2b). It seemed likely from these results that both the integrated and transiently expressed transgenes were targets of PTGS in the infiltrated leaf.

If transient expression is limited by PTGS, we expected that a viral suppressor of silencing would enhance the ectopic transgene expression in non-transgenic leaves. This prediction was confirmed, as shown in Figure 2(c), because GFP fluorescence in the presence of p25 (right panel) was stronger than in leaves co-infiltrated with water (left panel). Accordingly, there was more GFP in extracts of p25-treated leaves than in a −p25 control, as indicated by Western blot analysis (Figure 2d).

Other viral-encoded silencing suppressors (Brigneti *et al.*, 1998; Voinnet *et al.*, 1999) were also able to enhance the transient expression of GFP in non-transgenic *N. benthamiana*. However, the most pronounced effect was, by far, with the p19 protein (Figure 1) of TBSV (Voinnet *et al.*, 1999). At 5 days post-infiltration (dpi), the GFP fluorescence in the presence of p19 (Figure 2e) was brighter than with the PVX p25 (Figure 2c). Western blot analysis confirmed that GFP levels in the presence of p19 (Figure 2f) were substantially higher than in the absence of suppressor or in our GFP-expressing stable transgenic lines (*N. benthamiana* lines 8 and 16c; Ruiz *et al.*, 1998; Voinnet *et al.*, 1998; Figure 2f). Using a purified GFP standard, we estimated that this enhanced expression level corresponds to 270–340 μg GFP g^{-1} fresh tissue or approximately 7% of total soluble protein (Meristem Therapeutics, personal communication). In the absence of p19, there was only 5–12 μg GFP g^{-1} fresh tissue.

The second most effective suppressor after p19 was HcPro of potato virus Y (PVY; Brigneti *et al.*, 1998). For comparison, the Western blot in Figure 2(g) shows the GFP levels when the HcPro (35S:HcPro; Figure 1) was used instead of p19 in the transient expression assay. Clearly, the GFP enhancement caused by p19 was not only stronger than that of HcPro, it was also more persistent because the level of GFP protein remained high between 5 and 12 dpi with p19, whereas with HcPro it declined over this period. The effect of p19 persisted until 20 dpi when the onset of senescence in the infiltrated patch precluded further analysis (data not shown).

A time-course analysis confirmed that p19 strongly enhanced the level and stability of the ectopically

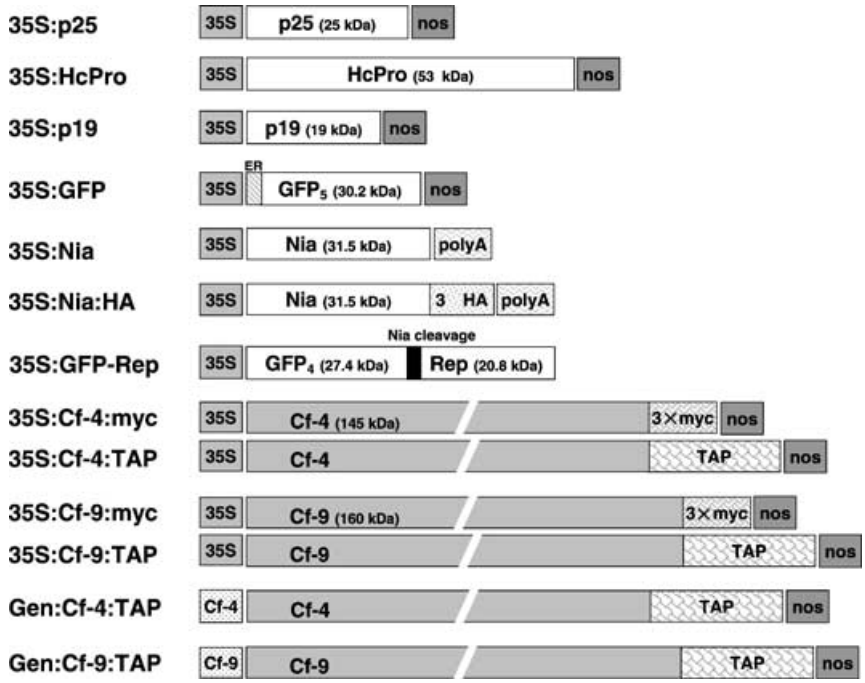

Figure 1. T-DNA constructs used in this study.
The predicted molecular weight of the corresponding proteins is indicated in brackets. The 35S:GFP construct carries an amino-terminal endoplasmic reticulum (ER) retention signal. The 35S:GFP-Rep is a translational fusion between the GFP_4 cDNA and the first 546 nucleotides of the potato virus X (PVX) replicase gene (Rep). The consensus cleavage site of the potato virus Y (PVY) NIa proteinase (NIa cliv.) has been inserted between the GFP_4 and Rep ORFs. Gen:Cf-4 and Gen:Cf-9 contain the Cf-4 and Cf-9 cDNA, respectively, cloned downstream of their cognate genomic promoter isolated from tomato. 35S: CaMV 35S promoter; Nos: nopaline synthase terminator; polyA: poly adenylation consensus signal; 3 × HA: triple hemagglutinin epitope tag; 3 × myc: triple c-myc epitope tag; TAP: tandem affinity purification tag. The nucleotide sequence of these peptides was inserted to create carboxy-terminal translational fusions, where indicated. All these constructs were based on the T-DNA of the pBin19 binary vector.

expressed GFP mRNA (Figure 2h, top panel). We also monitored the accumulation of the 21–25nt GFP siRNA (Figure 2h, lower panel) that is diagnostic of PTGS (Hamilton and Baulcombe, 1999). In tissues infiltrated in the absence of p19, the GFP siRNA was abundant and accumulated for at least 10 dpi (Figure 2h, bottom panel) corresponding to the decline in GFP mRNA levels (Figure 2h, top panel). In contrast, in all the p19 samples, the GFP siRNA was below the detection limit in all samples (Figure 2h, bottom panel) and GFP mRNA was abundant. This analysis therefore confirmed that the effect of p19 on GFP levels was primarily due to strong suppression of PTGS targeted against the transiently expressed GFP mRNA.

Agrobacterium-mediated transient expression of several other diverse proteins was also enhanced by p19. For example, an HA epitope-tagged NIa protease from PVY (Mestre *et al.*, 2000) (35S:NIa:HA; Figure 1) was detected by Western blot analysis at higher levels (>50-folds) at 4 dpi in the p19-treated tissues than in the controls (Figure 3a, left panel). Using an anti-HA affinity matrix, the NIa:HA protein could be readily purified from total soluble proteins extracted from p19-treated tissues (Figure 3a, right panel). As assessed on Coomassie-stained SDS–PAGE, the eluate contained a single protein component with an electrophoretic mobility corresponding to a molecular weight of 34 kDa that was specifically recognised by an anti-HA antibody (Figure 3a, right panel, red arrow). This protein was not detected in samples that had not been treated with p19. As little as a single *N. benthamiana*-infiltrated leaf (70–100 mg) was required for the purification and detection by Coomassie blue staining of the transiently expressed NIa:HA protein (Figure 3a, right panel, red arrow).

We also monitored the effect of p19 on transient expression of the tomato Cf-9 and Cf-4 glycoproteins conferring resistance to races of the fungus *Cladosporium fulvum* (Jones *et al.*, 1994; Thomas *et al.*, 1997). The Cf proteins were tagged with either the c-myc (Piedras *et al.*, 2000) or the TAP epitope (Rivas *et al.*, 2002; Figure 1). A time-course analysis revealed that, irrespective of whether the 35S or genomic promoters were used (Figure 3b), there was a low level of the tagged Cf protein at 1.5 and 2 dpi, both in the absence (left panel) or presence (right panel) of p19. Between 3 and 5 dpi, the Cf proteins increased in abundance in the presence of p19 (right panel) but were not detectable in the samples without p19 (left panel). Using a dilution series (data not shown), we estimate that p19 caused a fivefold enhancement in the abundance of the

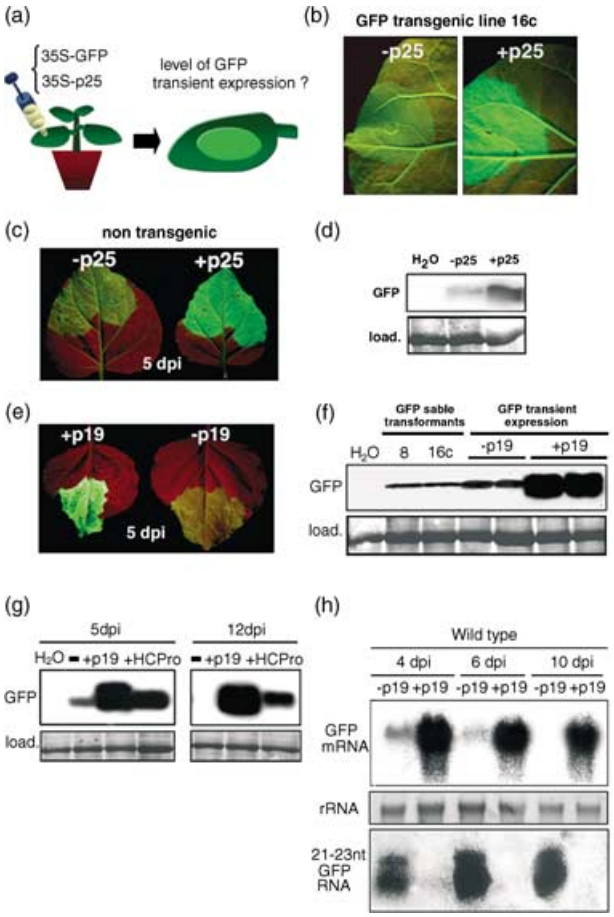

Figure 2. Effect of the p25 and p19 proteins on GFP transient expression.
(a) Individual *Agrobacterium* cultures carrying the 35S:GFP and the 35S:p25 constructs were mixed together and infiltrated into leaves of GFP transgenic *Nicotiana benthamiana* line 16c.
(b) Co-expression of p25 enhances ectopic GFP expression, as assessed at 5 days post-infiltration (dpi) under UV illumination.
(c) p25 also enhances ectopic GFP expression in leaves of wild-type *N. benthamiana*. The red background is due to chlorophyll fluorescence.
(d) Leaf discs from samples depicted in (c) were harvested and total solubilised protein extracts were prepared. Proteins were separated by SDS–PAGE and analysed by immunoblot using a GFP-specific antibody. Coomassie staining of total proteins indicates equal loading.
(e) Strong ectopic GFP expression elicited by the p19 protein in leaves of wild-type *N. benthamiana,* at 5 days post-infiltration (dpi).
(f) Compared levels of GFP expression between two stable *N. benthamiana* GFP transformants (high expressing lines 8 and 16c), and two independent samples from similar non-transgenic tissues in which transient expression of GFP was performed in the absence (–p19) or in the presence (+p19) of p19. Immunoblot analysis was as described in (d).
(g) Compared effect of the potato virus Y (PVY)-encoded HcPro and of p19 on ectopic GFP expression at 5 and 12 dpi. The experiment was as described in (c) and immunoblot analysis was as described in (d).
(h) Samples were collected for GFP mRNA and siRNA analysis. RNA was extracted from the entire infiltrated area at 4, 6 or 10 dpi. The high- (containing mRNA) and low-molecular weight (containing siRNA) fractions were separated by denaturing agarose and polyacrylamide gel electrophoresis, respectively. The agarose gels were stained with ethidium bromide to display relative amounts of rRNA, blotted and probed with ^{32}P-labelled GFP cDNA to detect GFP mRNA. The polyacrylamide gels were blotted and hybridised with ^{32}P-labelled GFP sense RNA to detect antisense siRNA. Hybridisation signals were detected by phosphorimaging.

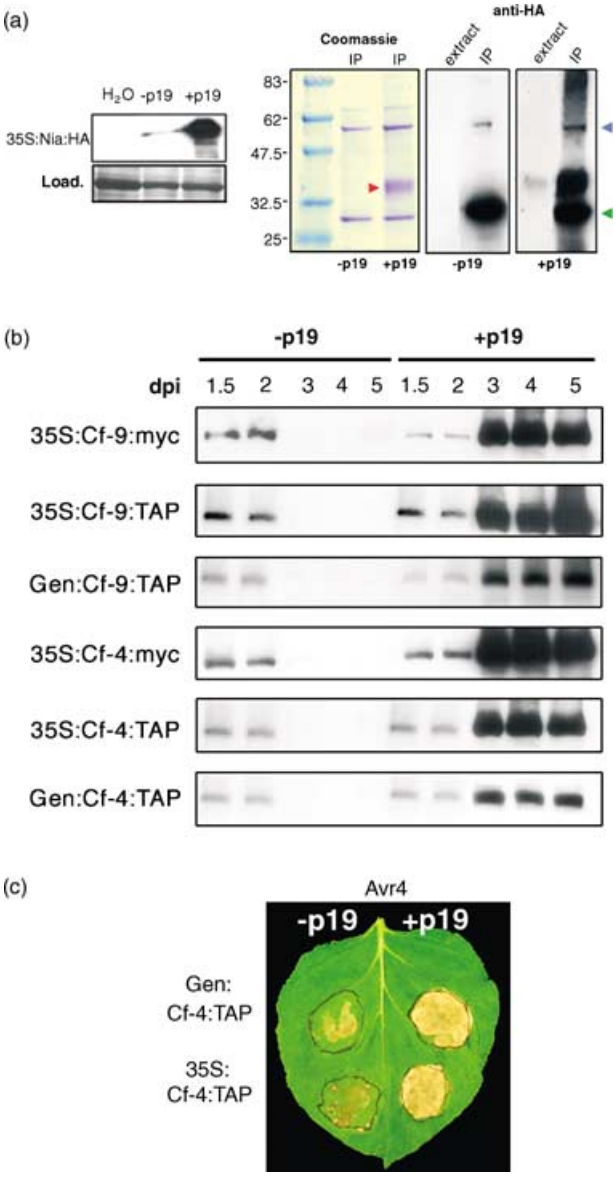

Figure 3. The p19 protein allows high-level expression of a protease and of Cf proteins.
(a) Transient expression of the 35S:Nla:HA in the presence of p19. The principle of the experiment is the same as in Figure 2(e). Individual leaf discs were sampled at 5 days post-infiltration (dpi), and total proteins were extracted. Immunoblot analysis was as described in Figure 2(d), except that an anti-HA-specific antibody was used (left panel). Protein extracts corresponding to 10 leaf discs (approximately 100 mg fresh tissue) were immunoprecipitated using an anti-HA affinity matrix (right panel). After Coomassie staining of the acrylamide gel, Nla:HA (red arrow) was detected in the immunoprecipitates (IP) only in the presence of p19. The heavy (blue arrow) and light (green arrow) chains of the antibody were also detected. Western blot analysis of the immunoprecipitates with an anti-HA antibody revealed strong expression of Nla:HA in the +p19 sample as compared to −p19. The light chain of the antibody (green arrow) was preferentially detected in the immunoblot because the antirat antibody used for detection was raised against rat Kappa and Lambda light chains.
(b) Enhanced transient expression of tagged Cf proteins in the presence of p19. *Agrobacterium* cells carrying the indicated Cf tagged constructs were mixed together with (+p19) or without (−p19) the 35S:p19 strain and infiltrated into *Nicotiana benthamiana* leaves. At the times indicated,

Cf proteins. The absence of a p19 effect in the first 2–3 dpi has been observed with other constructs (data not shown) and likely reflects the lag time necessary for synthesis of biologically active levels of p19.

Use of the *Agrobacterium* infiltration assay to express Cf-4 in transgenic plants expressing the *C. fulvum* Avr4 polypeptide results in chlorosis of the infiltrated area by 3–4 dpi and patches of necrosis at later times (Piedras *et al.*, 2000; Van der Hoorn *et al.*, 2000) (Figure 3c, left side of the leaf). It is thought that these responses are based on the signalling pathways that are activated naturally in the Avr-4-dependent, Cf-4-mediated disease resistance (Jones *et al.*, 1994; Thomas *et al.*, 1997). When this assay was performed in the presence of p19, the Cf-4-dependent cell death was accelerated by 4 days and covered the entire infiltrated zone (Figure 3c, right side of the leaf). Therefore, we conclude that p19 caused an increase in expression of biologically active Cf-4.

It was striking that the p19-enhanced expression of GFP and Cf4-mediated HR was manifested uniformly across the infiltrated region of the leaf (Figures 2e and 3c). In principle, this effect could be due to intercellular movement of the overexpressed proteins. However, if the 35S:GFP *Agrobacterium* strain was diluted so that the T-DNA was transferred into isolated cells within the infiltrated region, the p19-mediated enhancement of GFP expression was restricted to single cells (Figure 4a,b). It is likely, therefore, that there is a high incidence of T-DNA co-transfer, as reported previously (Kapila *et al.*, 1997; Vaquero *et al.*, 1999), in the cells of the infiltrated region.

To further investigate the potential for simultaneous expression of multiple proteins, we infiltrated *N. benthamiana* leaves with three strains of *Agrobacterium* carrying 35S:GFP, 35S:Cf9-myc and 35S:NIa:HA constructs (Figure 1) either with or without the 35S:p19 strain (Figure 4c). The amount of these proteins produced at 4 dpi was estimated by Western blot analysis and compared to the amount produced when p19 was used with

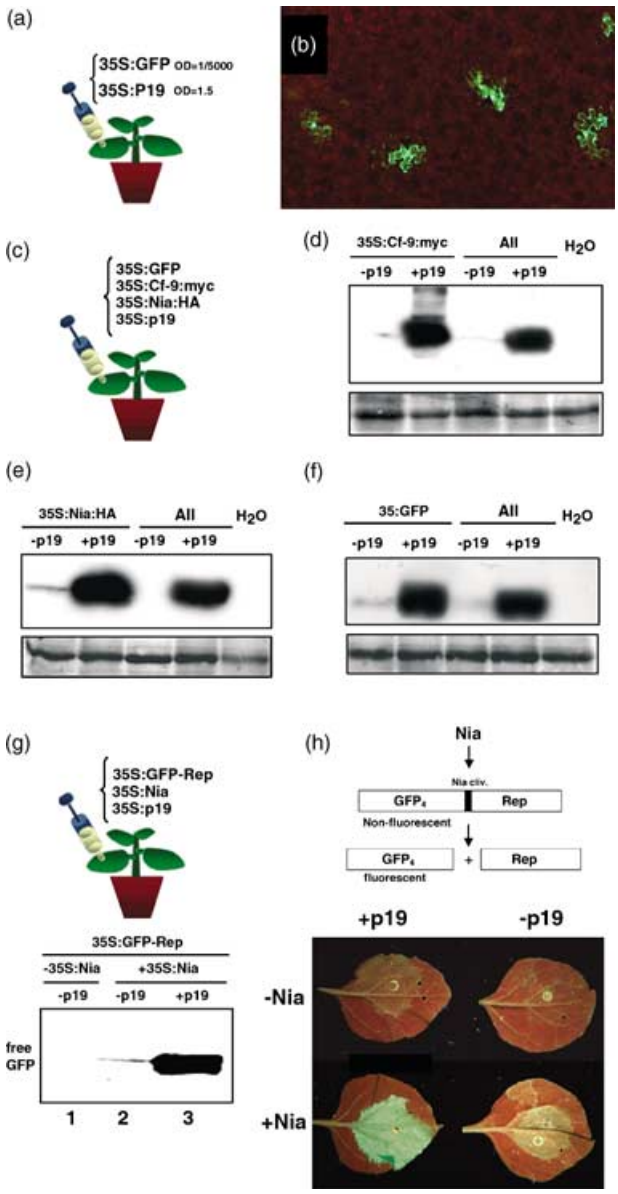

Figure 3. continued
samples were harvested and total solubilised protein extracts were prepared. Proteins (50 μg) were separated by SDS–PAGE and analysed by immunoblot using a PAP or anti-c-myc antibody for detection of TAP- and c-myc-tagged Cf-9, respectively.
(c) The Cf-4/Avr-4-dependent hypersensitive cell death is accelarated and enhanced in the presence of p19. *Agrobacterium* carrying the indicated TAP-tagged Cf-4 constructs were mixed together with (+p19) or without (−p19) the 35S:p19 strain, and infiltrated into transgenic *N. benthamiana* leaves expressing Avr4. After 5 days, the Cf-4/Avr-4-dependent hypersensitive cell death reaction was monitored. The edges of infiltrated patches were highlighted with a black marker pen. Similar results were obtained when the Cf-9/Avr-9-dependent cell death was assayed (data not shown).

Figure 4. Use of p19 for enhanced expression of multiple proteins.
(a) The principle of these experiments is the same as in Figure 2(e), except that the culture of the *Agrobacterium* strain 35S:GFP ($OD_{600} = 1.0$) was diluted 5000 times in infiltration buffer prior to mixing with the 35S:p19 strain ($OD_{600} = 1.5$).
(b) Samples were collected at 5 days post-infiltration (dpi) and observed under a dissecting microscope coupled to an epi-fluorescence module. Enhanced GFP expression is confined into individual cells (epidermal, here). Under the same conditions, single cell GFP expression is almost undetectable in samples that have not been treated with p19 (data not shown).
(c) Individual cultures of *Agrobacterium* strains 35S:GFP, 35S:Cf-9:myc, 35S:NIa:HA were mixed all together (All) with (+p19) or without (−p19) the 35S:p19 strain. For reference, individual cultures were also mixed with a control *Agrobacterium* strain carrying a 35S:GUS transgene (data not shown), with or without the p19 strain.
(d) Immunoblot analysis of Cf-9:myc expression using an anti-myc antibody.
(e) Immunoblot analysis of NIa:HA using an anti-HA antibody.
(f) Immunoblot analysis of GFP using a plolyclonal GFP antibody.
(g–h) Co-expression of the 35:GFP-Rep and 35S:NIa constructs. The Rep sequence in 35S:GFP-Rep prevents fluorescence of the fusion protein, either in the presence (+p19; panel 1 in photograph) or absence (−p19; panel 2 in photograph) of the 35S:p19 strain. Only NIa-mediated cleavage of the fusion protein would release fluorescent, free GFP. This event occurs at a low level when the 35S:NIa strain is added to the infiltration mix (+NIa; panel 3 in photograph; lane 2 of immunoblot) and is strongly enhanced by the co-expression of p19 (+NIa; panel 4; lane 3 of immunoblot).

individual constructs. Figure 4(d–f) indicates that a similar increase in protein levels was observed irrespective of whether GFP, Cf9-myc and NIa:HA were expressed singly or in combination. Thus antisilencing activity of p19 was not saturated in cells that had received up to four individual T-DNA constructs.

Co-expression of NIa protease of PVY and a GFP fusion protein (Figure 4g,h) also illustrated the potential of the system for simultaneous expression of interacting proteins. The GFP was fused at the carboxy terminus to part of a viral replicase protein. This fusion protein was not fluorescent under UV light. However, as the fusion protein has a NIa cleavage site immediately at the carboxy terminus of the GFP sequence, co-expression of the protease and the fusion protein results in release of fluorescent GFP (Figure 4h, diagram). If the overexpressed NIa protease, the fusion protein and p19 were in different cells, the presence of p19 would not result in an increase in released GFP. However, as the co-expression of NIa and p19 caused a large and uniform increase in the level of fluorescent GFP (Figure 4g,h), the three proteins must have been present in the same cells and able to interact.

Discussion

Based on the present study, it is clear that *Agrobacterium*-mediated transient gene expression is limited by PTGS and that this limitation can be overcome by virus-encoded suppressor proteins. These findings extend a previous report (Johansen and Carrington, 2001) by showing that PTGS is a general feature of transient expression and p19 is a more effective suppressor than HcPro. We also show that transient expression in the presence of suppressors is useful for enhanced expression of a range of proteins expressed either individually or in mixtures. In principle, this *in planta* expression could be the first step in protein purification and biochemical analysis, as shown for the NIa:HA protease (Figure 3a). The p19 protein may also be useful in combination with viral and/or transgenic systems for high-level protein expression (Mallory *et al.*, 2002).

We anticipate that enhanced transient expression could be easily scaled-up as a rapid and cost-effective expression system for a large variety of plant and foreign proteins. It may be particularly useful when post-translational modifications are required for biological activity, as illustrated here with the Cf-4 and Cf-9 glycoproteins (Figure 3b). Yields may be further enhanced by targeting products to the apoplast or other cellular compartments where they are protected from degradation.

The ability to enhance simultaneously the expression from several T-DNA constructs is a particular attraction of the system (Figure 4). It will allow efficient engineering of complex metabolic pathways as well as multimeric proteins. In stable transgenic plants such manipulation would require sequential transformations or crosses between transgenic plants that could take months, if not years.

Functional genomics/proteomics approaches could also greatly benefit from the procedure described here. The most straightforward application is in rapid gene discovery, whereby a candidate protein would be assessed functionally *in planta*. The long-lasting effect of p19 also makes it possible to analyse the long-term effects of wild-type or engineered proteins on global gene expression, metabolic pathways or changes in subcellular structures. In addition, the use of p19 will likely facilitate the rapid and large-scale assessment of protein variants generated, for instance, by DNA shuffling.

In the present analysis, the host plant was *N. benthamiana*. However, the p19 suppressor is effective in *Arabidopsis* (OV, unpublished data), and it is likely that similar approaches could be developed in other species that are amenable to *Agrobacterium*-mediated or other transient expression systems. Other viral suppressor proteins that are adapted to the other plants of interest may also be useful, although none has been identified so far that is as effective as p19. The recent identification of a suppressor of silencing from an insect-infecting virus may indicate that the approach described here could also be developed for overexpression in animals (Li *et al.*, 2002).

Besides its practical significance, the work reported here also prompts a number of fundamental questions. First, what are the factors that influence activation of PTGS in the infiltrated leaves? We can rule out an effect of the promoter of the transiently expressed gene because there was PTGS with both 35S and Cf promoters. A more likely explanation is at the RNA level. Presumably, transient gene expression is inevitably associated with aberrant transcription that would produce a dsRNA activator of PTGS. Alternatively, the aberrant RNA could be a template for an RNA-dependent RNA polymerase (Dalmay *et al.*, 2000; Mourrain *et al.*, 2000) that would synthesise the dsRNA. A second issue is the possibility that PTGS against transgenes in the T-DNA is recapitulating a defence reaction against *Agrobacterium* that is normally targeted against the bacterial oncogenes. If that is the case, it may be expected that the bacterium has evolved counter-defence strategies and, like viruses, would encode PTGS-suppressor proteins (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau and Carrington, 1998; Voinnet, 2001; Voinnet *et al.*, 1999).

Experimental procedures

Plants

Wild-type *N. benthamiana* and the GFP transgenic lines 16c and 8 were grown as previously described (Ruiz *et al.*, 1998; Voinnet *et al.*, 1998).

Agroinfiltration procedure

Agrobacterium tumefaciens strain C58C1 was grown at 29°C in L-broth supplemented with 50 μg ml^{-1} kanamycin and 5 μg ml^{-1} tetracycline to stationary phase. Bacteria were sedimented by centrifugation at 5000 ***g*** for 15 min at room temperature and re-suspended in 10 mM $MgCl_2$ and 150 μg ml^{-1} acetosyringone. Cells were left in this medium for 3 h and then infiltrated into the abaxial air spaces of 2–4-week-old *N. benthamiana* plants. All the *Agrobacterium* strains harboured the pCH32 helper plasmid (Hamilton *et al.*, 1996). The culture of *Agrobacterium* carrying the p25 construct was brought to an optical density (OD_{600}) of 1.0 to avoid toxicity (Voinnet *et al.*, 2000). Transient co-expression of the Cf and p19 constructs was at OD_{600} of 0.1 and 1.0, respectively.

GFP imaging

The GFP fluorescence was monitored by epi-illumination with a hand-held UV source, as described (Voinnet *et al.*, 1998). A dissecting microscope (Leica MZ-FLIII), coupled to an epifluorescence module, was used for single cell observations.

DNA constructs

The 35S:p25 and 35S:GFP_5 constructs were described previously (Voinnet *et al.*, 2000). The 35S:p19 and 35S:HcPro constructs were made by inserting PCR-amplified fragment of the p19 and HcPro DNA (Brigneti *et al.*, 1998; Voinnet *et al.*, 1999) into *Sma*I-linearised pBin61 (Bendahmane *et al.*, 2000). The 35S:NIa construct was described previously (Mestre *et al.*, 2000). The 35S:NIa:HA construct was obtained as follows. A triple HA tag was amplified from plasmid pACTAG-2 and ligated to a PCR-amplified, 3′ fragment of the NIa protease ORF (249nt). Primer sequences can be obtained on request. PCR products were gel-purified, ligated, and the ligation product was cloned directionally into *Stu*I-*Xma*I-linearised pBINYPro (Mestre *et al.*, 2000). The 35:GFP-Rep construct was obtained by chimaeric PCR as follows. The GFP_4 ORF was amplified from plasmid mGFP4 (Haseloff *et al.*, 1997) using primers GFPSal (5′-TTCTAGGTCGACATGAGTAAAGGAGAAGAAC-3′) and GFPtgt (5′-GTCATTTCCTTGATGGTGCACTTCATATTTGTATAGTTCATCC-3′). The PVX RdRp sequence corresponding to the first 182 amino acids was amplified from pTXS (Kavanagh *et al.*, 1992) using primers REPtgt (5′-TATGAAGTGCACCATCAAGGAAATGAC1AGGTGCGCG-3′) and REP*Xma*I (5′-GCTTTCCCCGGGTTAGGCTGCCTCAACGGG-3′). GFPtgt and REPtgt contain the nucleotide sequence of the NIa protease cleavage site (VHHQG). Both PCR products were gel-purified, mixed and subjected to five cycles of PCR without primers; then, primers GFP*Sal*I and REP*Xma*I were added and further 25 PCR cycles were performed. The resulting product was gel-purified, digested with *Sal*I and *Xma*I and cloned directionally into pBINY53 (Mestre *et al.*, 2000). The various 35S:Cf derivatives were described previously (Piedras *et al.*, 2000; Rivas *et al.*, 2002).

RNA extraction, Northern analysis

Total RNA was extracted using Tri-Reagent (Sigma, St. Louis, MI, USA) according to the manufacturer's instructions. High- and low-molecular weight RNAs were fractionated and analysed according to Voinnet *et al.* (2000).

Protein extraction, Western blot analysis and protein purification

For GFP extraction, leaf discs (one cap of an Eppendorf tube) were ground into 200 μl extraction buffer (4 M urea, 100 mM DTT). Hundred microlitres of loading buffer (Laemmli, 1970) were added and the samples were boiled for 5 min and subsequently centrifuged at 10 000 ***g*** for 10 min at 4°C. Twenty-five microlitres of the supernatant was then loaded on a 12% SDS gel (Laemmli, 1970). Proteins were separated and transferred onto nitrocellulose by wet electroblotting. For detection of GFP, a mouse monoclonal GFP antibody (BD Clontech no. 8371-1) and an antimouse antibody conjugated to peroxidase (Sigma) were used at 1 : 5000 and 1 : 10 000 dilutions, respectively. Blots were developed using the ECL kit (Pierce, Rockford, IL, USA) and chemiluminescence emitted from the filter was quantified directly with a Fluoro image analyser (FLA-5000, Fujifilm, Tokyo, Japan). A dilution series of purified recombinant GFP was used as an internal standard. The voltage applied to the photo-multiplier was 500 V.

The extraction and detection of the Cf and NIa:HA proteins were as described previously (Piedras *et al.*, 2000; Rivas *et al.*, 2002). The extracts were filtered through two layers of Miracloth. After centrifugation at 1000 ***g*** for 10 min at 4°C, the supernatant was recovered and subsequently ultracentrifuged at 100 000 ***g*** for 1 h at 4°C. This high-speed supernatant was subjected to immunoprecipitation with anti-HA affinity matrix (Roche, Manheim, Germany). Ten microlitres of matrix were used per 25 μg of total soluble protein. After 2 h at 4°C, the beads were washed three times with extraction buffer and proteins were separated on a 10% SDS gel and transferred onto nitrocellulose by wet electroblotting. For detection of NIa:HA, a rat monoclonal anti-HA antibody (Roche, clone 3F10) and an antirat antibody conjugated to peroxidase (Sigma) were used at 1 : 2000 and 1 : 10 000 dilutions, respectively. Blots were developed using the ECL kit (Pierce).

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