

## TECHNICAL ADVANCE

# Agrodrench: a novel and effective agroinoculation method for virus-induced gene silencing in roots and diverse Solanaceous species

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## Summary

Virus-induced gene silencing (VIGS) is an extremely powerful tool for plant functional genomics. We used *Tobacco rattle virus* (TRV)-derived VIGS vectors expressed from binary vectors within *Agrobacterium* to induce RNA silencing in plants. Leaf infiltration is the most common method of agroinoculation used for VIGS but this method has limitations as it is laborious for large-scale screening and some plants are difficult to infiltrate. Here we have developed a novel and simple method of agroinoculation, called 'agrodrench', where soil adjacent to the plant root is drenched with an *Agrobacterium* suspension carrying the TRV-derived VIGS vectors. By agrodrench we successfully silenced the expression of phytoene desaturase (*PDS*), a 20S proteasome subunit (*PB7*) or Mg-protoporphyrin chelatase (*Chl H*) encoding genes in *Nicotiana benthamiana* and in economically important crops such as tomato, pepper, tobacco, potato, and Petunia, all belonging to the Solanaceae family. An important aspect of agrodrench is that it can be used for VIGS in very young seedlings, something not possible by the leaf infiltration method, which usually requires multiple fully expanded leaves for infiltration. We also demonstrated that VIGS functioned to silence target genes in plant roots. The agrodrench method of agroinoculation was more efficient than the leaf infiltration method for VIGS in roots. Agrodrench will facilitate rapid large-scale functional analysis of cDNA libraries and can also be applied to plants that are not currently amenable to VIGS technology by conventional inoculation methods.

**Keywords:** agrodrench, virus-induced gene silencing, agroinoculation, *Tobacco rattle virus*, Solanaceae.

## Introduction

Virus-induced gene silencing (VIGS) is an RNA-mediated post-transcriptional gene-silencing mechanism that protects plants against foreign gene invasion (Baulcombe, 1999). In addition to allowing a better understanding of how plants defend themselves against plant viruses, VIGS has emerged as a functional genomics tool for knocking out gene expression of target plant genes in some plants (Ekengren *et al.*, 2003; Holzberg *et al.*, 2002; Liu *et al.*, 2002a; Peart *et al.*, 2002; Sharma *et al.*, 2003; Yoshioka *et al.*, 2003). VIGS is also being used as a forward genetics

tool to identify a desired phenotype (Lu *et al.*, 2003b). Plant virus-based vectors carrying plant sequences homologous to endogenous plant genes trigger gene silencing through a homology-dependent RNA degradation mechanism commonly referred to as RNA silencing. Small interfering (si) RNA, derived from targeted dsRNA of virus or host origin and expressed from the viral genome, would target the degradation complex to the corresponding host mRNA resulting in a loss-of-function phenotype in the host (Abbink *et al.*, 2002; Baulcombe, 2002; Lu *et al.*, 2003a;

Robertson, 2004; Turnage *et al.*, 2002; Voinnet, 2001). In contrast to conventional mutagenesis, VIGS does not alter the gene itself, but rather transiently suppresses the expression of the gene through degradation of mRNA transcripts. Thus VIGS allows, unlike stable transformation procedures, the study of genes that would normally lead to a lethal phenotype when disrupted (Kim *et al.*, 2003b; Lu *et al.*, 2003a).

Among the several viral vector systems used to trigger VIGS, *Tobacco rattle virus* (TRV)-derived vectors are widely used because they produce mild symptoms on the host and TRV has a wide host range (Dinesh-Kumar *et al.*, 2003; Liu *et al.*, 2002a; Ratcliff *et al.*, 2001). TRV belongs to the genus Tobravirus and is mostly transmitted by nematodes through the soil (Matthews, 1991; Visser and Bol, 1999). TRV contains bipartite positive-sense RNA genome (RNA1/RNA2; Matthews, 1991). RNA1 encodes two viral replication proteins, a movement protein and a seed transmission factor. RNA2 encodes the coat protein and a nematode transmission factor (Verchot-Lubicz, 2002; Visser and Bol, 1999).

*Agrobacterium tumefaciens* is a soil-borne pathogen that causes neoplastic growth, referred to as 'crown gall,' in several dicotyledonous plants by entering mainly through wounds on roots and stem (Agrios, 1997; Burr and Otten, 1999; Escobar and Dandekar, 2003). *Agrobacterium*-based binary vectors have been widely used for delivering viral vectors into plants and this is referred to as agroinoculation (Evans and Jeske, 1993; Hanley-Bowdoin *et al.*, 1988; Rochester *et al.*, 1990). Agroinoculation is also extensively used to deliver VIGS vectors into plants for RNA silencing (Gosselé *et al.*, 2002; Liu *et al.*, 2002b; Peart *et al.*, 2002). Leaf infiltration is the most common method of agroinoculation used for VIGS (Dinesh-Kumar *et al.*, 2003; Lu *et al.*, 2003a). However, the leaf infiltration method has limitations. Leaf infiltration is laborious for large-scale screening and certain plants, such as soybean and maize, are difficult to infiltrate. Leaf infiltration of TRV-based VIGS vectors do not induce efficient RNA silencing in many plants including varieties of tomato (Ekengren *et al.*, 2003; Liu *et al.*, 2002a). Leaf infiltration also normally utilizes fully expanded leaves (Liu *et al.*, 2002b; Ratcliff *et al.*, 2001). These limitations prevent the efficient use of VIGS technology on some plant species and all young seedlings.

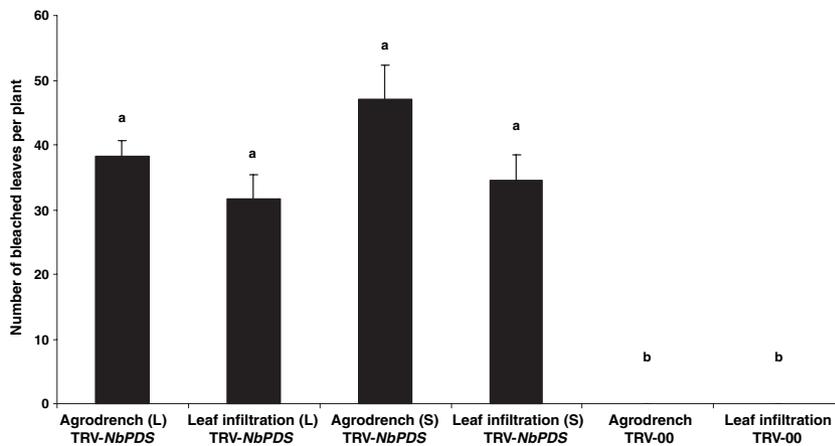
Here we report the development and use of a novel method of agroinoculation, called 'agrodrench' that addresses some of the problems normally associated with agroinoculation during VIGS. We have demonstrated that agrodrench with a TRV-VIGS vector can be used for RNA silencing in diverse Solanaceae species and in young seedlings. Interestingly, agrodrench increases the efficacy of VIGS in roots compared with leaf infiltration. We propose that agrodrench is a simple and effective agroinoculation technique that can be extensively used for infecting a wide range of plants to induce VIGS.

## Results and discussion

### *Agrodrench is as effective as leaf infiltration method of agroinoculation for VIGS in Nicotiana benthamiana*

Although the leaf infiltration method of agroinoculation works efficiently in *N. benthamiana*, a popularly used plant for VIGS experiments, it can be a laborious process for large-scale screens using VIGS. To decrease the labor required during forward genetic screens we developed a simple and efficient method of agroinoculation by drenching the plant rhizosphere (crown part of plant) with *A. tumefaciens* containing the viral vector within the T-DNA of a binary vector. We used TRV [bipartite RNA virus; TRV-RNA1 (TRV1) and TRV-RNA2 (TRV2)] as a VIGS vector. This virus has already been used for VIGS in *N. benthamiana* (Liu *et al.*, 2002b; Lu *et al.*, 2003b; Ratcliff *et al.*, 2001), a miniature tomato cultivar, referred to as Micro-tom (Liu *et al.*, 2002a; Scott and Harbaugh, 1989) and recently in potato (Brigneti *et al.*, 2004).

To test the effectiveness of agrodrench in *N. benthamiana*, we used a *N. benthamiana* phytoene desaturase (*NbPDS*) gene in TRV2 cDNA contained within the T-DNA of an *Agrobacterium* (strain GV2260) binary vector. The *PDS* gene encodes an enzyme involved in carotenoid biosynthesis and silencing of this gene results in photobleaching of plant leaves (Kumigai *et al.*, 1995). *Agrobacterium* strains containing TRV1 and TRV2 (containing *NbPDS*) were induced with acetosyringone and were mixed in a 1:1 ratio and applied directly onto the soil adjacent to the crown part of 2–3-week-old *N. benthamiana* plants. Photobleaching was observed on the newly developed leaves of *N. benthamiana* plants after 10 days of inoculation. We optimized the agrodrench procedure by using several *Agrobacterium* concentrations (containing a mixture of *Agrobacterium* strains with TRV1 and TRV2-*NbPDS* in 1:1 ratio) and found that the minimum concentration required to elicit VIGS was  $10^2$  colony forming units (cfu) ml<sup>-1</sup>. However, at lower *Agrobacterium* concentrations (< $10^4$  cfu ml<sup>-1</sup>), appearance of gene-silencing phenotype was delayed by 6–8 days and also reduced the frequency of silencing to 20–30% in *N. benthamiana*. The minimum concentration of *Agrobacterium* required to obtain 100% frequency of gene silencing by agrodrench was  $10^6$  cfu ml<sup>-1</sup>. However, at this concentration the results were not consistent from independent experiments. We consistently obtained a gene-silencing frequency of 100% in *N. benthamiana* with *Agrobacterium* concentrations of  $10^8$  cfu ml<sup>-1</sup> and above. We did not observe any harmful effects caused by high concentrations ( $10^{10}$ – $10^{12}$  cfu ml<sup>-1</sup>) of *Agrobacterium* by agrodrench (data not shown). *Agrobacterium* cultures grown on solid medium were slightly more effective in inducing VIGS when compared with liquid-grown cultures, although they



**Figure 1.** Comparison of agrodrench and leaf infiltration methods of agroinoculation.

*Nicotiana benthamiana* plants were inoculated with *Agrobacterium* solutions made from cells grown on solid medium (S) or liquid medium (L), containing *pTRV2* alone (TRV-00) or TRV2-*NbPDS* (TRV-*NbPDS*) either by agrodrench or leaf infiltration methods. *Agrobacterium*-containing *pTRV1* was co-inoculated along with *Agrobacterium*-containing *pTRV2*. Number of bleached leaves per plant in each treatment was counted 21 days after TRV inoculation. Experiment was conducted four times with six replications in each experiment. Letters indicate significant differences using Fisher's LSD test at  $P = 0.05$ .

were not statistically different at  $P = 0.05$  (Figure 1). Viral transcripts were detected in the photobleached leaves through reverse transcriptase-polymerase chain reaction (RT-PCR; data not shown).

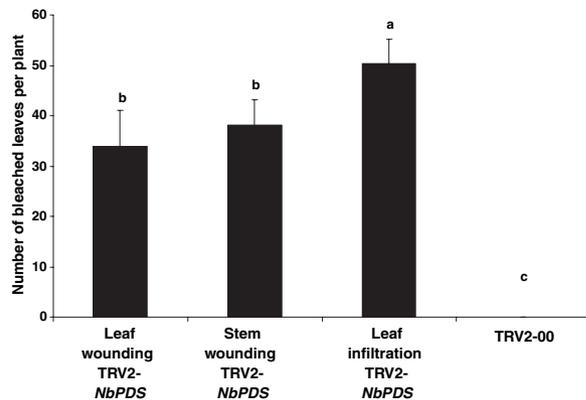
We compared the frequency of VIGS by agrodrench method with the commonly used leaf infiltration method. The frequency of VIGS was determined by the number of plants that show silencing phenotype (photobleaching) after inoculation with TRV2-*NbPDS*. By both agrodrench and leaf infiltration methods of agroinoculation, 100% of the infected *N. benthamiana* plants showed the silencing phenotype. The efficacy of VIGS was determined by counting each and every leaf (small and big), including leaves of secondary shoots, that showed photobleaching. Our results, shown in Figure 1, indicate that the agrodrench method was slightly more effective in VIGS than that of the leaf infiltration method of agroinoculation. However, statistically the number of silenced leaves between the two methods was not significantly different at  $P = 0.05$  (Figure 1). The only difference we found between the two methods was that the appearance of silencing phenotype was delayed by 3–4 days in agrodrench method when compared with the leaf infiltration method. The delay in silencing may be due to the length of time required for the virus or the silencing signal to move from the site of infection (roots) to the upper parts of the plants (leaves).

Because agrodrench is an unconventional method of agroinoculation, we determined whether transformation-efficient *Agrobacterium* was required for the silencing. To determine whether *Agrobacterium* transformation was required for the agrodrench method of agroinoculation, we transferred the TRV1 and TRV2-*NbPDS* vectors into an avirulent *Agrobacterium* strain that does not contain a Ti

plasmid and inoculated *N. benthamiana* using the agrodrench method. Three weeks after inoculation we did not see photobleaching on plants infected with the avirulent *Agrobacterium* strain (data not shown). This result suggests that *Agrobacterium*-mediated T-DNA transformation is required for agrodrench method of agroinoculation. We speculate that the developing plant roots mimic wounding through which *Agrobacterium*-mediated transformation occurs.

#### *Agrodrench can be utilized for large-scale VIGS experiments using a fast-forward genetics approach*

Virus-induced gene silencing is an excellent tool for high-throughput fast-forward genetics (Lu *et al.*, 2003b; Robertson, 2004). Using this approach it was recently shown that a heat-shock protein 90 plays a critical role in plant disease resistance (Lu *et al.*, 2003b). Fast-forward genetics using TRV vectors normally involves infiltrating freshly grown *Agrobacterium* strain containing TRV1, using a needle-less syringe, to leaves of 3–4-week-old *N. benthamiana* plants. Clones from 96-well microtiter plates containing TRV2 (contains cDNA library) are individually picked with a toothpick and then pricked to the area of the leaf infiltrated with TRV1 (Olga del Pozo and Greg Martin, BTI, Ithaca, New York, USA, personal communication; C.-M. Ryu and K. S. Mysore unpublished data). Leaf infiltration of TRV1 to hundreds of plants is laborious. We used agrodrench method to apply TRV1 to *N. benthamiana* plants. TRV1 alone, in the absence of TRV2, moved systemically in plants (Ratcliff *et al.*, 2001). One week after TRV1 application, *Agrobacterium* (grown on solid medium) containing TRV2-*PDS* was picked with a toothpick and inoculated to either the stem or the leaf



**Figure 2.** Feasibility of using agrodrench for large-scale experiments. *Nicotiana benthamiana* plants were inoculated with *Agrobacterium*-containing *pTRV1* by agrodrench method. One week after TRV1 inoculation, *Agrobacterium* containing *pTRV2* alone (TRV-00) or TRV2-*NbPDS* (TRV-*NbPDS*) was inoculated either by wounding the stem or leaf with toothpick or infiltrating the leaf with a needle-less syringe. Number of bleached leaves per plant in each treatment was counted 21 days after TRV2 inoculation. Experiment was conducted two times with nine replications in each experiment. Letters indicate significant differences using Fisher's LSD test at  $P = 0.05$ .

of *N. benthamiana* plants. Liquid grown *Agrobacterium* containing TRV2-*NbPDS* was also applied by the leaf infiltration method. Photobleaching was observed on the upper leaves of all the plants inoculated with TRV2-*NbPDS* (Figure 2). These results suggest that the agrodrench method can be effectively used for high throughput TRV-vector-based VIGS experiments by minimizing the manpower.

#### Agrodrench is more effective than the leaf infiltration method of agroinoculation for VIGS in roots

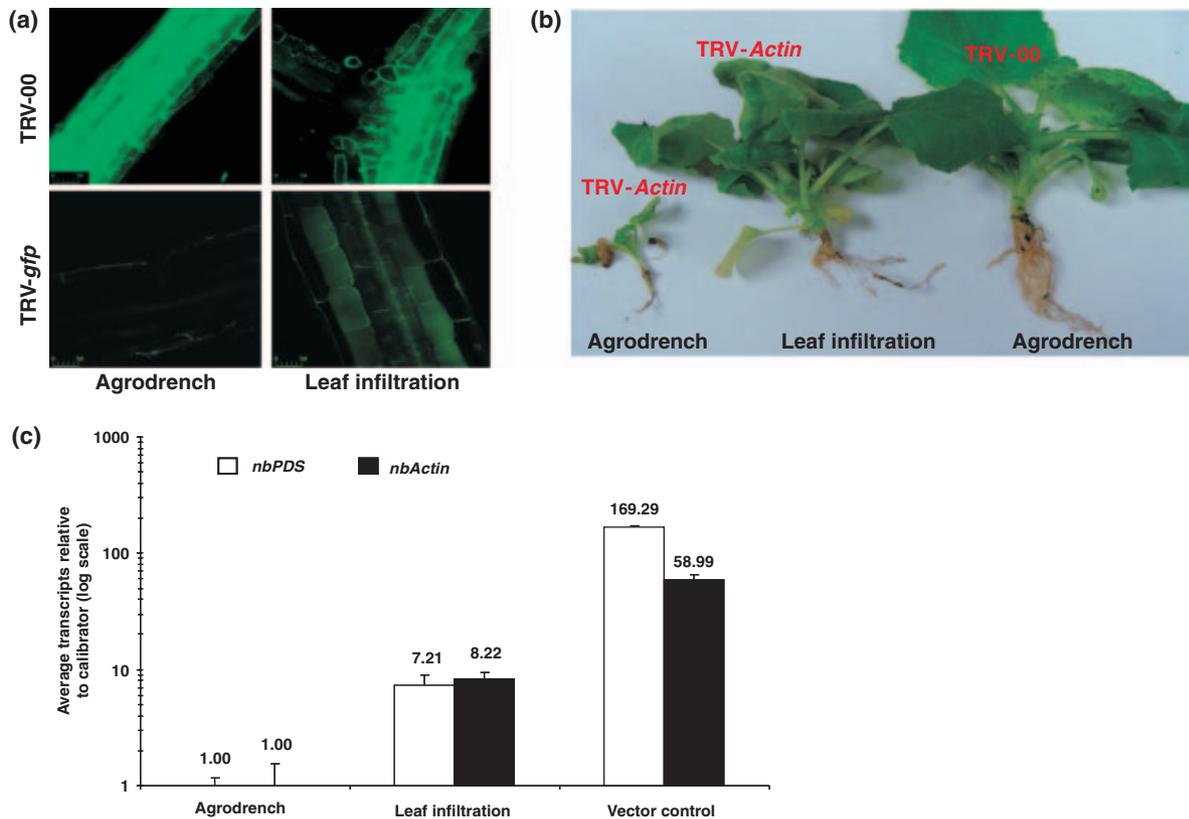
Virus-induced gene silencing is popularly used to transiently knockout genes that show a characteristic phenotype on shoot tissue (Abbink *et al.*, 2002; Burger *et al.*, 2003; Ekegren *et al.*, 2003; Liu *et al.*, 2002b). TRV-based VIGS was recently shown to be effective in silencing genes in plant roots (Saedler and Baldwin, 2004). TRV is a soil-borne virus transmitted by nematodes and accumulates to high titer in the root system (MacFarlane and Popovich, 2000; Verchot-Lubicz, 2002). Therefore, we hypothesized that root infection with TRV-VIGS vectors will be more effective than leaf infection for silencing genes in roots. We compared both leaf infiltration and agrodrench methods of agroinoculation to determine the efficacy of VIGS in plant roots by using green fluorescent protein (*gfp*), *PDS* and *Actin* as indicator genes. *Agrobacterium* containing TRV2-*NbPDS* or TRV2-*NbActin* was inoculated, along with *Agrobacterium* containing TRV1, onto wild-type *N. benthamiana* plants either by leaf infiltration or agrodrench methods. *Agrobacterium* strain containing TRV2-*gfp*, along with *Agrobacterium* containing TRV1, was inoculated on to 16C plants (transgenic *N. benthamiana*

plant expressing *gfp*; Brigneti *et al.*, 1998) either by leaf infiltration or agrodrench methods. *Agrobacterium* containing TRV2-empty vector was used as a control. Gene-silencing phenotypes were observed in the roots of TRV2-*gfp* and TRV2-*NbActin* inoculated plants 14 days after inoculation (Figure 3a,b). Silencing of the *NbActin* gene resulted in severely stunted shoot and roots and silencing of *gfp* gene, in 16C plants, resulted in loss of green fluorescence under UV light. Interestingly, the agrodrench method caused a more severe phenotype when compared with the leaf infiltration method. We speculate that the accumulation of the virus in the roots following root transformation contributes to more rapid and effective VIGS in roots compared with the leaf infiltration method where the virus or the gene-silencing signal (Ruiz *et al.*, 1998) must move from the leaves to the root to trigger gene silencing.

To further confirm gene silencing at the molecular level, we performed quantitative RT-PCR analyses to quantify the transcript levels of the silenced endogenous genes (Figure 3c). RNA was isolated at 14 days after inoculation, from roots of TRV2-*NbPDS*, TRV2-*NbActin* and TRV2-empty vector-inoculated plants. Gene-specific primers for *NbPDS* and *NbActin* were used to amplify the respective genes through quantitative RT-PCR. Suppression of *PDS* and *Actin* transcripts was slightly more effective using agrodrench method of VIGS when compared with leaf infiltration (Figure 3c). These findings reveal the potential for functional characterization of genes expressed in roots that are involved in diverse biological functions (physiological, biochemical, and plant-microbe interactions).

#### Agrodrench is effective for VIGS in various plant species within the Solanaceae family

*Nicotiana benthamiana* is a member of the Solanaceae, a widely distributed family of plants to which many economically important species belong, including tomato, potato, pepper, eggplant, tobacco, and Petunia. Although TRV can systemically move in many Solanaceae species, TRV-based VIGS has been demonstrated only in *N. benthamiana*, *N. attenuata*, tomato, and potato (Brigneti *et al.*, 2004; Liu *et al.*, 2002a,b; Ratcliff *et al.*, 2001; Saedler and Baldwin, 2004). We applied agrodrench method of VIGS to different Solanaceae species such as tomato, tobacco, pepper, Petunia, potato, and eggplant using *PDS* and *PB7* (encodes  $\beta 7$  subunit of 20S proteasome) as indicator genes. Using TRV2-*NbPDS* we successfully silenced the endogenous *PDS* orthologs of tomato, tobacco, and Petunia indicating that the *PDS* gene sequences are sufficiently conserved among these species to allow silencing (Figure 4a; Figure S1). Surprisingly, TRV2-*NbPDS* did not silence the *PDS* orthologs of pepper, potato, and eggplant. We then PCR amplified the *PDS* gene of pepper (*pPDS*) and cloned into TRV2-VIGS vector to determine the efficiency of silencing in pepper.



**Figure 3.** RNA silencing in roots by agrodrench method.

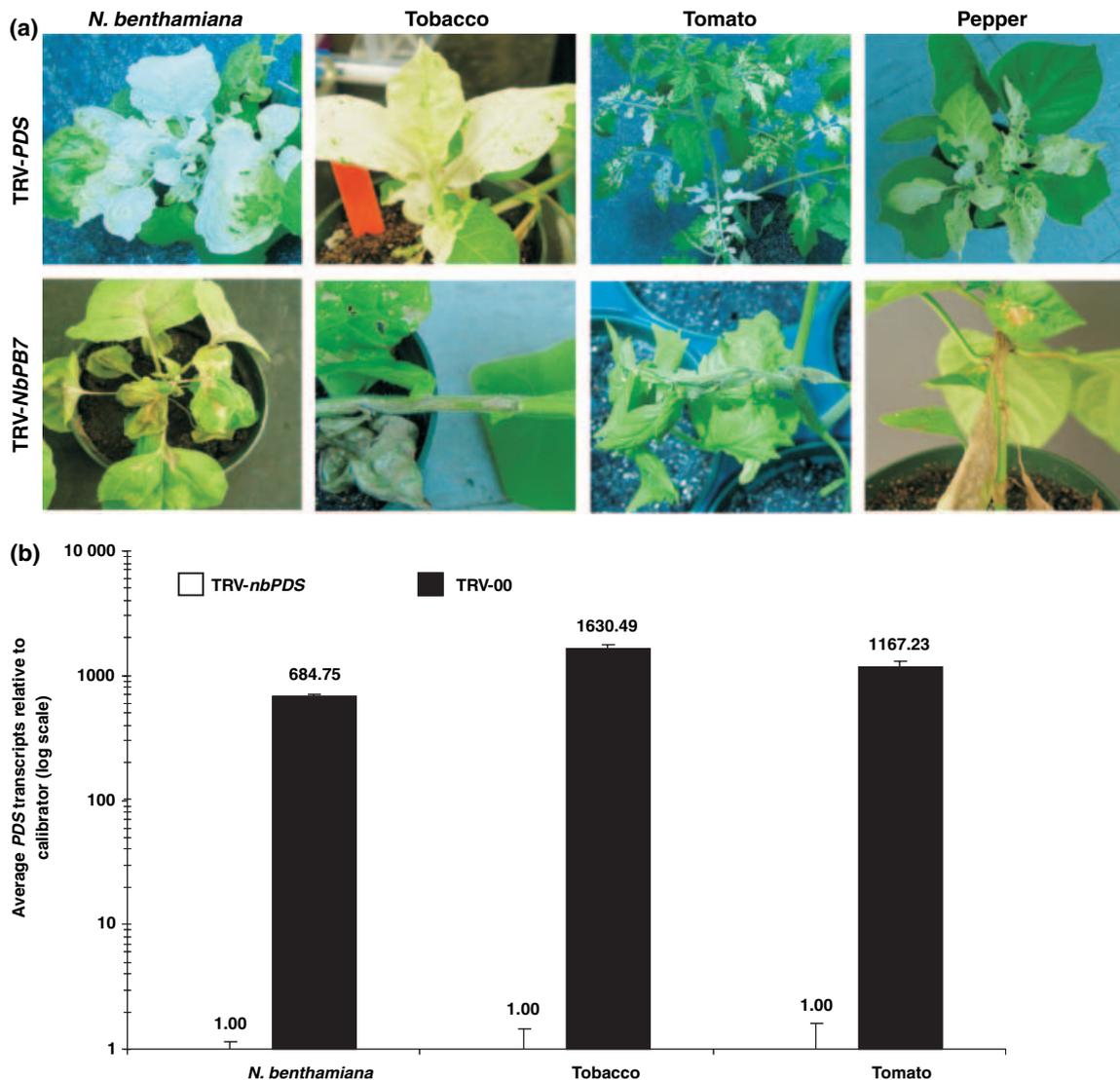
(a) Suppression of *gfp* expression in the roots of *Nicotiana benthamiana* 16C plants. GFP fluorescence in roots was observed under confocal microscope 14 days after infection with TRV-*gfp* or TRV-00 (empty vector) by agrodrench or leaf infiltration. Bar = 50  $\mu$ m.

(b) Suppression of *Actin* gene in *N. benthamiana* roots. *Nicotiana benthamiana* plants were inoculated with *Agrobacterium* containing TRV-*Actin* or TRV-00 (empty vector) either by agrodrench or leaf infiltration method. Photograph was taken 14 days after inoculation.

(c) Virus-induced gene silencing-mediated degradation of specific RNA transcripts in *N. benthamiana* roots. Total RNA was isolated from roots of the *NbPDS*- and *NbActin*-silenced plants, either by leaf infiltration or agrodrench method, and was used to generate first-strand cDNA. RNA from plants inoculated with TRV alone was used as a vector control. cDNA was used for quantitative PCR reaction using primers specific to the targeted gene. The amount of elongation factor-1- $\alpha$  transcripts was determined for every gene-silenced plant as a control (data not shown) and was used for normalization of *PDS* and *Actin* transcripts. The amount of *PDS* and *Actin* gene transcripts from the agrodrench-silenced plants were used as calibrators to obtain the relative fold differences. Note that the agrodrench method of gene silencing is more effective in degrading specific root transcripts when compared with the leaf infiltration method.

TRV2-*pPDS* was able to successfully silence the endogenous pepper *PDS* by agrodrench method of inoculation (Figure 4a). Interestingly, the TRV2-*pPDS* was not able to silence *PDS* ortholog of *N. benthamiana*. TRV2-tomato *PDS* (*tPDS*) inoculation on potato also did not show any photo-bleaching on potato leaves. Further experiments have to be performed to determine why orthologous gene sequences do not cause silencing in related plant species. By using agrodrench method of agroinoculation, the *PDS* silencing was observed in 100% of infected *N. benthamiana* plants while only around 60–70% of the infected plants showed *PDS* silencing phenotype in other Solanaceae species. The relative abundance of *PDS* transcripts in some of the silenced plants was determined by quantitative RT-PCR (Figure 4b). The primers used for RT-PCR were designed to specifically amplify transcripts of *PDS* orthologs in *N. benthamiana*, tomato, and tobacco.

20S proteasome is one of the subunits of the 26S proteasome complex involved in protein degradation by the ubiquitin pathway (Sullivan *et al.*, 2003; Vierstra, 2003). 20S proteasome is a stack of four seven-membered rings, the two outer rings being formed by seven  $\alpha$  subunits and the two inner rings by seven  $\beta$  subunits (Coux, 1996). Previous study has demonstrated that the suppression of *NbPAF* (encodes  $\alpha 6$  subunit of 20S proteasome) expression by VIGS resulted in spontaneous programmed cell death in *N. benthamiana* accompanied by reduced proteasome activity and accumulation of polyubiquitinated proteins (Kim *et al.*, 2003a,b). Due to the obvious phenotype induced by silencing of 20S proteasome subunit, we selected this for VIGS analysis. *PB7* encodes the  $\beta 7$  subunit of the 20S proteasome complex and a partial gene sequence of this gene has been reported for tobacco (*NtPB7*; Dahan *et al.*, 2001). We cloned a partial gene sequence of *PB7* from *N. benthamiana* (*NbPB7*)



**Figure 4.** Application of agroinfiltration method for virus-induced gene silencing in different Solanaceae species.

(a) *PDS* and *PB7* silencing in *Nicotiana benthamiana*, tomato, tobacco and pepper. Two to 3-week-old plants were inoculated with *Agrobacterium* containing either TRV-*PDS* or TRV-*NbPB7* by agroinfiltration method. Photograph was taken 21 days after inoculation. Experiment was conducted three times with 10 replications for each experiment.

(b) Degradation of *PDS* transcripts during gene silencing in various Solanaceae species. Total RNA was isolated from leaves of the various plants silenced for *PDS*, by agroinfiltration method, and was used to generate first-strand cDNA. RNA from plants inoculated with TRV alone (TRV-00) was used as a control. cDNA was used for quantitative PCR reaction using primers specific to *PDS* gene (see Experimental procedures). The amount of elongation factor-1 $\alpha$  transcripts was determined for every gene-silenced plant as a control (data not shown) and was used for normalization of *PDS* transcripts. The amount of *PDS* gene transcripts from each silenced plant was individually used as calibrator to obtain the relative fold differences.

into TRV2-VIGS vector (see Experimental procedures) and we inoculated TRV2-*NbPB7* onto several Solanaceae species by agroinfiltration. Two weeks after inoculation, strong systemic necrosis phenotype was observed on *N. benthamiana*, tobacco, tomato, and pepper (Figure 4a). Milder necrotic symptoms were observed on potato leaves (Figure S1). The pattern of cell death depended on plant species. *Nicotiana benthamiana* and tomato developed systemic necrosis in the meristem that progressed downward, but tobacco and

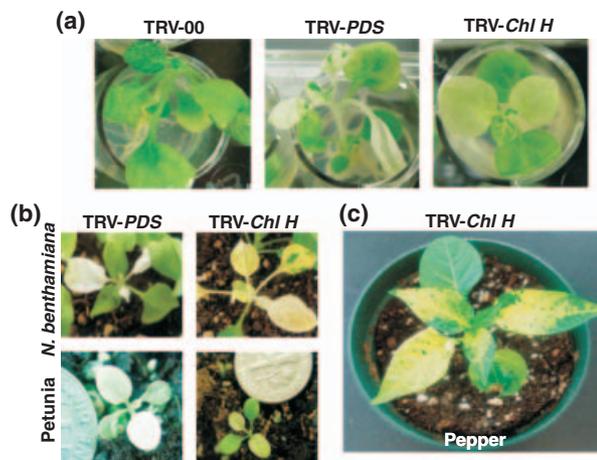
pepper showed severe symptoms on the stem and leaves while potato showed milder cell death on the leaves (Figure S1). Interestingly the eggplant did not show a silencing phenotype with TRV2-*NbPDS* or TRV2-*NbPB7* inoculation by agroinfiltration. More studies are required to determine whether the transformation and the virus movement in eggplant were as efficient as in other Solanaceae species. The *NbPB7* silencing was more efficient (90–100%) in producing visible symptoms when compared with *PDS*

silencing. We propose that *NbPB7* silencing is a better visual indicator of VIGS when compared with *PDS* silencing.

*Agrodrench is an excellent method to elicit VIGS in very young seedlings*

One of the advantages of VIGS is that it can be applied to mature plants to assess the function of genes, whose mutation (or antisensing) might be lethal in sexually propagated plants (Kim *et al.*, 2003b; Lu *et al.*, 2003a). However, current VIGS protocols are limited in assessing the function of genes in young seedlings or during seedling development. Leaf infiltration method of VIGS is normally applied to 3–4-week-old seedlings having at least two true leaves. Maximum gene silencing is normally observed 2 weeks after agroinoculation and by that time the age of the plant is at least 5–6 weeks. We have used the agrodrench method of VIGS to silence specific genes in 1–2-week-old young seedlings. *PDS*, *PB7*, and *Chl H* genes were used as visual indicators of gene silencing. *Chl H* encodes the H subunit of magnesium protoporphyrin chelatase, an enzyme involved in chlorophyll biosynthesis, popularly known as the sulfur (*Su*) gene (Hudson *et al.*, 1993; Kruse *et al.*, 1997). Reduction or absence of magnesium protoporphyrin chelatase in plants will result in yellow-colored leaves because of reduction in chlorophyll synthesis (Hiriart *et al.*, 2002, 2003). Occurrence of the visual phenotype of *Chl H* silencing is much faster than *PDS* silencing in *N. benthamiana* (C.-M. Ryu and K. S. Mysore, unpublished data). Therefore, *Chl H* silencing is more sensitive and a better visual indicator of gene silencing than *PDS* silencing and hence we used *Chl H* as one of the indicators for gene silencing in young seedlings.

*Nicotiana benthamiana Chl H* gene was PCR amplified from *N. benthamiana* leaf cDNA and cloned into TRV2-VIGS vector. Agrodrench method was used to individually inoculate *Agrobacterium* strains containing either TRV2-*NbChl H* or TRV2-*NbPDS* or TRV2-*NbPB7* on freshly germinated 1-week-old soil-grown seedlings of *N. benthamiana*, pepper, Petunia, and tomato. We also applied the agrodrench method of agroinoculation for plants grown under sterile conditions. *Agrobacterium* strains containing either TRV2-*NbChl H* or TRV2-*NbPDS* were inoculated, by dropping 50  $\mu$ l of bacterial suspension at the crown part of the 1-week-old seedling on the sterile media on which *N. benthamiana* seedlings were germinated. Seven to 10 days after inoculation, yellowing, photobleaching and cell death phenotypes were observed for plants inoculated with TRV2-*NbChl H*, TRV2-*NbPDS* and TRV2-*NbPB7* respectively (Figure 5; Figure S1). Interestingly, application of agrodrench method directly to seeds or germinating seeds failed to elicit VIGS. Minimum age of plant at which agrodrench can be applied to achieve gene silencing was 1 week. These results indicate that agrodrench is an effective method for VIGS in very young seedlings.



**Figure 5.** Gene silencing in very young seedlings.

(a) Silencing of *PDS* and *Chl H* genes in sterile grown *Nicotiana benthamiana* seedlings. *Nicotiana benthamiana* seeds were surface-sterilized and germinated on sterile growth media (see Experimental procedures) in 24-well microtiter plates. One week after germination, *Agrobacterium* containing TRV-00 (empty vector) or TRV-*NbPDS* or TRV-*NbChl H* was applied on to the agar medium along with *Agrobacterium* containing *pTRV1*. One week after inoculation, photobleaching was observed for *PDS*-silenced plants and yellowing was observed for *Chl H*-silenced plants.

(b and c) Silencing of *PDS* and *Chl H* genes in soil-grown *N. benthamiana*, Petunia and pepper seedlings. Seeds were germinated in soil and 1 week after germination, *Agrobacterium* containing either TRV-*NbPDS* or TRV-*NbChl H*, along with *Agrobacterium* containing *pTRV1*, was applied by the agrodrench method. One week after inoculation, photobleaching was observed for *PDS*-silenced plants and yellowing was observed for *Chl H*-silenced plants. Photograph was taken 2 weeks after inoculation. Experiment was conducted three times with 10 replications for each experiment.

The agrodrench method of VIGS in very young seedlings will open a new research area to study the role of certain genes during seedling development in both soil and sterile system.

## Conclusions

Virus-induced gene silencing is an excellent functional genomics tool for gene function analyses. We have developed, agrodrench, a novel and simple agroinoculation method to elicit VIGS. As the agrodrench and leaf infiltration procedures were similar in efficiency, the ease of the agrodrench method suggests it would be a better choice for high-throughput gene silencing and for plants which are difficult to infiltrate. Agrodrench method can be used for efficient VIGS in various Solanaceae species. We used the *PDS*, *PB7*, and *Chl H* genes as indicators of gene silencing in foliar tissue. *PB7* and *Chl H* are more sensitive indicators of gene silencing than *PDS*. We have demonstrated that agrodrenching shuts down transcript levels to a greater degree in roots than did leaf infiltration. This may allow us to do gene functional analyses during root–microbe interactions such as plant–*Rhizobium* symbiosis, plant–mycorrhizal symbiosis, plant–rhizobacteria, and plant–pathogen associations.

## Experimental procedures

### Plant material and growth conditions

*Nicotiana benthamiana* [both wild-type and 16C transgenic plants expressing *gfp* (Brigneti *et al.*, 1998)], tomato (*Lycopersicon esculentum* cvs. Rutgers and Micro-Tom), bell pepper (*Capsicum annuum* cv. Marengo), tobacco (*Nicotiana tabacum* cv. Xanthi nc), potato (*Solanum tuberosum* cv. Catalina), eggplant (*Solanum melongena* cv. Louisiana Long Green), and Petunia (*Petunia hybrida* cv. Freedom Red Star) were used for our experiments. Seeds were germinated in flats with a soil-less potting mixture, BM7 (Berger Co., Quebec, Canada). Two-week-old seedlings were transplanted to 10 cm diameter round pots, containing BM7, with one plant per pot. Fertilizer (20-10-20) along with soluble trace element mix (The Scotts Co., Marysville, OH, USA) was applied with water. Greenhouse conditions were kept at  $23 \pm 3^\circ\text{C}$  and 70% humidity under 16 h extended day with supplemental lighting with  $50\text{--}100 \mu\text{E sec}^{-1} \text{m}^{-2}$  light intensity. One to 3-week-old plants were used for silencing experiments. To obtain sterile *N. benthamiana* plants, seeds were surface-sterilized with 70% ethanol for 1–2 min followed by treatment with 1% sodium hypochlorite for 20 min. Seeds were washed several times with sterile water and was plated on 24-well microtiter plates (one seed/well) containing MS agar [ $4.32 \text{ g l}^{-1}$  Murashige and Skoog (MS) minimal salts, 1.5% sucrose, and 1% phytoagar] and incubated at  $24^\circ\text{C}$  with 12 h light.

### Plasmid construction

*pTRV1* and *pTRV2* VIGS vectors (described in Liu *et al.*, 2002a) were obtained from Dr Dinesh-Kumar, Yale University. A 430-bp pepper *PDS* fragment was amplified by RT-PCR with primers, *pPDSattB1*: 5'-ggggacaagttgtacaaaaagcaggctGCAATGGAAGGAACATTCGA-3' and *pPDSattB2*: 5'-ggggaccactttgtacaagaagctgggtAGTCTCTCAGGAGATTACC-3', from common bell pepper. A 451-bp *gfp* fragment was amplified using primers, *gfpattB1*: 5'-ggggacaagttgtacaaaaagcaggctCTTTTCACTGGAGTTGTC-3' and *gfpattB2*: 5'-ggggaccactttgtacaagaagctgggtGCTTGTGGCCATGATGTA-3', from *N. benthamiana* 16C plants. A 136-bp *PB7* gene (encodes a  $\beta 7$  subunit of 20S proteasome) fragment was amplified by RT-PCR using primers, *Nb20PattB1*: 5'-ggggacaagttgtacaaaaagcaggctCATGCCATTCACGGAG-3' and *Nb20PattB2*: 5'-ggggaccactttgtacaagaagctgggtGACTTGGTGGAGTGAAAAATGGAC-3', from *N. benthamiana* plants. A 365-bp *NbChl H* gene fragment was amplified by RT-PCR using primers *NbChlHattB1*: 5'-ggacaagttgtacaaaaagcaggctCGAGCGCCGCCGGCAGGTGGAGATGT-3' and *NbChlHattB2*: 5'-ggggaccactttgtacaagaagctgggtCATGAATTGAGCTTGAACCTTGCCATTGT-3', from *N. benthamiana* plants. The PCR-amplified gene fragments were introduced into GATEWAY ready *pTRV2* (Liu *et al.*, 2002a) by using GATEWAY cloning system according to the manufacturer's recommendations (Invitrogen Co., Carlsbad, CA, USA). *TRV2-NbActin* was obtained from Dr Jianzhong Liu and Dr Rick Nelson, Noble Foundation (Liu *et al.*, unpublished data). *TRV2-NbPDS* and *TRV2-tPDS* (Ekengren *et al.*, 2003) were obtained from Dr Greg Martin, BTI, Cornell University. Sequence of all the *pTRV2* derivatives were confirmed by sequencing at the Noble Foundation. Plasmids were introduced into *A. tumefaciens* strain GV2260 by electroporation.

### RNA extraction and RT-PCR analysis

Total RNA from leaf and root tissue were isolated from silenced and non-silenced (infiltrated with empty vector *pTRV1* and *pTRV2*)

plants 3 weeks post-inoculation using TRIzol® reagent (Invitrogen Co.) according to the manufacturer's manuals and treated with RNase-free DNase (RQ1; Promega, Madison, WI, USA). An RT reaction was performed on 1–5  $\mu\text{g}$  of total RNA with 200 units of SuperScript™ RNase H-RT (Invitrogen Co.), 250 ng of gene-specific primers and 500  $\mu\text{M}$  dNTPs in a final volume of 20  $\mu\text{l}$ . Gene-specific primers in the RT reaction were designed using the PrimerQuest software for RT-PCR from IDT (Integrated DNA Technologies, Coralville, IA, USA). Real-time PCR was performed with ABI PRISM 7000 (ABI; Applied Biosystems Inc., Foster City, CA, USA) using SYBR Green (ABI). The primers used for quantifying the *PDS* gene from tobacco, *N. benthamiana*, and tomato were 5'-TAAACCCTGACGAGCTTTCGA-3' and 5'-TTGTGTTTGGCCACTCCAGTGGA-3'. The primers used to quantify the *Actin* gene from *N. benthamiana* were 5'-TGGTCGTACCACCGGTATTGTGTT-3' and 5'-TCACTTGCCCATCAGGAAGCTCAT-3'. For the relative quantification of the gene transcripts in different species, standard curve method (separate tubes) was applied according to the manufacturer's protocol (ABI User Bulletin no. 2). As a control for silenced and non-silenced plants, parallel reactions using *N. benthamiana* elongation factor 1- $\alpha$  (*NbEF1  $\alpha$* ) primers were carried out and the data obtained were used to normalize *PDS* and *Actin* transcripts from various Solanaceae species. The primers used to amplify a fragment of the *NbEF1  $\alpha$*  gene from *N. benthamiana* were 5'-ACGTGAGCGTGGTATCACATT-3' and 5'-ATCAGGACAGCACAGTCAGCTT-3'. Each sample was run in triplicate and repeated twice from pooled samples of three independently silenced and non-silenced plants. The average of the two experiments was calculated, and the results were graphed with the corresponding standard deviation indicated with error bars in the figures.

### Agrodrench and leaf infiltration methods

The *A. tumefaciens* strain GV2260 containing TRV-VIGS vectors was used for VIGS experiments. Bacteria were grown at  $28^\circ\text{C}$  either on Luria-bertani (LB) agar medium or LB broth with appropriate antibiotics. The bacterial cells were harvested either by scraping the bacteria from agar medium (for bacteria grown on solid medium) or by centrifugation (for bacteria grown in liquid medium) and resuspended into *Agrobacterium* inoculation buffer (10 mM  $\text{MgCl}_2$ , 10 mM MES pH 5.6, 150  $\mu\text{M}$  acetosyringone) to a final  $\text{OD}_{600}$  of 1.0 (for both TRV1 and TRV2) and shaken for 4–6 h at room temperature before infiltration.

For leaf infiltration, each *Agrobacterium* strain containing TRV1 and TRV2 vectors were mixed in 1:1 ratio and infiltrated to the leaves of 2–3-week-old plants with a 1 ml needle-less syringe. For agrodrench, mixture of *Agrobacterium* strains containing TRV1 and TRV2 was drenched, 3–5 ml each, with a 10-ml pipette into the crown part of each plant. Accumulation of virus in the freshly grown part of the plant was detected, 2 weeks after inoculation, by PCR with TRV coat protein-specific primers 5'-CTGGGTTACTAGCGGC-CTGAATA-3' (forward primer) and 5'-TCCACCAAACCTTAATCCGAATAC-3' (reverse primer). Six to 10 replications were performed for each experiment and the experiment was repeated at least two times.

### Confocal microscopy

Seeds of *N. benthamiana* 16C plants stably expressing GFP (Brigneti *et al.*, 1998) were obtained from Dr David Baulcombe and planted as described above. Two weeks after inoculation of plants with *Agrobacterium* by leaf infiltration and agrodrench methods, we observed *N. benthamiana* roots with a Bio-Rad 1024 ES confocal laser

scanning microscope (Bio-Rad, Hercules, CA, USA). GFP in living roots was detected by exciting samples with the 488-nm line of the Krypton–Argon laser and capturing the emission at 522 nm. All images were processed using Adobe Photoshop 5.0 L.E. (Adobe Systems Inc., Mountain View, CA, USA).

#### Data analysis

Data were subjected to analysis of variance using JMP software version 4.0.4 (SAS Institute Inc., Cary, NC, USA). When a significant *F*-test was obtained at  $P = 0.05$ , separation of treatment means was accomplished by Fisher's protected least significant difference.

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#### Supplementary Material

The following material is available from <http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ2211/TPJ2211sm.htm>.

**Figure S1.** Silencing phenotypes of Petunia, potato, and tomato by agrodrench.

(a) *PDS* silencing in Petunia. Petunia plant was inoculated with *Agrobacterium* containing TRV2-*NbPDS* by agrodrench method. Photograph was taken 21 days after inoculation.

(b) *PB7* silencing in potato. Potato plant was inoculated with *Agrobacterium* containing TRV2-*NbPDS* by agrodrench method. Photograph was taken 21 days after inoculation.

(c) Silencing of *PB7* gene in soil grown tomato seedlings. Seeds were germinated in soil and 1 week after germination, *Agrobacterium* containing TRV2-*NbPB7*, along with *Agrobacterium* containing pTRV1, was applied by agrodrench method. One week after inoculation, cell death was observed in silenced plants. Photograph was taken 2 weeks after inoculation.

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