Electronic Journal of Biotechnology ISSN: 0717-3458
Vol.10 No.2, Issue of April 15, 2007

Received August 23, 2006 / Accepted November 9, 2006
DOI: 10.2225/vol10-issue2-fulltext-11
RESEARCH ARTICLE

A practical approach to the understanding and teaching of RNA silencing in plants

Ariel A. Bazzini
Instituto Nacional de Tecnología Agropecuaria
Instituto de Biotecnología
Las Cabañas y Los Reseros
B1712WAA Castelar, Argentina
Tel: 5411 4621 1447. Ext 146
Fax: 5411 4621 0199
E-mail: abazzini@cnia.inta.gov.ar

Vanesa C. Mongelli
Instituto Nacional de Tecnología Agropecuaria
Instituto de Biotecnología
Las Cabañas y Los Reseros
B1712WAA Castelar, Argentina
Tel: 5411 4621 1447. Ext 146
Fax: 5411 4621 0199
E-mail: vmongeli@cnia.inta.gov.ar

H. Esteban Hopp
Unidad Integrada de Investigación y Docencia
Centro Nacional de Investigaciones Agropecuarias
Facultad de Ciencias Exactas y Naturales
Universidad de Buenos Aires
Las Cabañas y Los Reseros
B1712WAA Castelar, Argentina
Tel: 5411 4621 1447. Ext 146
Fax: 5411 4621 0199
E-mail: ehopp@cnia.inta.gov.ar

Mariana del Vas
Instituto de Tecnología Agropecuaria
Instituto de Biotecnología
Las Cabañas y Los Reseros
B1712WAA Castelar, Argentina
Tel: 5411 4621 1447. Ext 146
Fax: 5411 4621 0199
E-mail: mdelvas@cnia.inta.gov.ar

Sebastián Asurmendi*
Instituto de Tecnología Agropecuaria
Instituto de Biotecnología
Las Cabañas y Los Reseros
B1712WAA Castelar, Argentina
Tel: 5411 4621 1447. Ext 146
Fax: 5411 4621 0199
E-mail: sasurmendi@cnia.inta.gov.ar

Financial support: This research was partially supported by grants PICT 11196, PID 267 and PAV 137 from FONCyT-ANPCyT and PIP 5788 from CONICET and other funding from INTA, Argentina.

Keywords: gene silencing, GFP, HC-Pro, PDS, protocols, PTGS, teaching, transgenic, transient expression, TRV, VIGS.

Abbreviations:
dsRNA: double stranded RNA
gfp: green fluorescent protein
PDS: phytoene desaturase
PTGS: Post-transcriptional gene silencing
RNAi: RNA interference
siRNA: short interfering RNAs
TRV: *Tobacco rattle virus*

*Corresponding author

Gene silencing, also called RNA interference (RNAi) is a specific mechanism of RNA degradation involved in gene regulation, development and defense in eukaryotic organisms. It became an important subject in the teaching programs of molecular biology, genetics and biotechnology courses in the last years. The aim of this work is to provide simple and inexpensive assays to understand and teach gene silencing using plants as model systems. The use of transient and permanent transgenic plants for expressing reporter genes, like those derived from jellyfish green fluorescent protein (*gfp*) encoding gene, provides a nice, colorful and conclusive image of gene silencing. Three experimental approaches to evidence RNA silencing are depicted. In the first approach gene silencing is demonstrated after transient expression of reporter genes in non-transgenic plants. In the second, silencing is triggered against a reporter gene stably integrated into a transgenic plant. The third approach involves the triggering of RNA silencing against endogenous genes using viral vectors. In addition we illustrate systemic gene silencing showing how the silencing signal is spread over a plant and finally it is also demonstrated the suppression of gene silencing. The first group of experiments is recommended to be tough on undergraduate courses, the following two sections are recommended for graduate courses. Hopefully, it will help students to understand this important phenomenon and to unravel the importance of gene silencing as a key gene regulation mechanism and as a molecular and biotechnological tool.

Post-transcriptional gene silencing (PTGS) in plants, known as RNA interference (RNAi) in animals, is an inducible and specific mechanism of RNA degradation (Baulcombe, 2004). It was shown that gene silencing is not only an important gene regulation mechanism but also a defense mechanism against foreign or aberrant endogenous RNAs, like virus and retrotransposons (Aravin et al. 2001; Hamilton et al. 2002). Due to the important role of RNA silencing *per se* and the multiple biotechnological uses that derived from the understanding of this phenomenon, we consider that this topic should be taught to biology students. We exemplify here a simple practical approach to the understanding and teaching of RNA silencing in plants.

Molecular basis of RNA silencing

Although RNA silencing operates through diverse pathways, it is most efficiently triggered by double stranded RNA (dsRNA) intermediates, which are processed into RNA duplexes of 21-24 nucleotides by a ribonuclease III-like enzyme called Dicer (Fire et al. 1998; Bernstein et al. 2001; Wesley et al. 2001). Once produced, these small RNA molecules or short interfering RNAs (siRNAs) are incorporated in a multi-subunit complex called RNA-induced silencing complex or RISC (Hammond et al. 2000; Tang et al. 2003). RISC is formed by a siRNA and an endonuclease, among other components. The siRNAs within RISC acts as a guide to target the degradation of complementary messenger RNAs (mRNAs) (Hammond et al. 2000; Tang et al. 2003). The summary of the silencing mechanism can be seen in Power Point Presentation 1. More recently, a closely related mechanism of RNAi was described for the regulation of the expression of endogenous genes including some involved in developmental processes. The host genome codify for small RNAs called microRNAs or miRNAs that are responsible of the endogenous gene silencing. The dsRNAs triggering of silencing can originate from several sources such as expression of endogenous or transgenic antisense sequences, expression of inverted repeated sequences or RNA synthesis during viral replication (Voinnet, 2005a). When dsRNA molecules produced during viral replication trigger gene silencing, the process is called virus-induced gene silencing (VIGS) (Lu et al. 2003). One interesting feature of RNA silencing in plants is that once is triggered in a certain cell, a mobile signal is produced and spread through the whole plant causing the entire plant to be silenced (Dunoyer et al. 2006).

RNA silencing and viral infections

It was proposed that being RNA silencing an antiviral defense mechanism, viruses must have evolved a counter-defense strategy (Mallory et al. 2001; Ratcliff et al. 2001). Consistent with this hypothesis, in the last 8 years several laboratories have discovered and characterized viral-encoded molecules with the capacity to suppress gene silencing (Brigneti et al. 1998; Anandalakshmi et al. 2000; Lucy et al. 2000; Zhou et al. 2006). Such molecules have also been found in a large number of plant and animal viruses (Anandalakshmi et al. 1998; Brigneti et al. 1998; Li et al. 2002). Nowadays, it is expected that practically most viruses carry suppressors of gene silencing, which are diverse in sequence, evolutionary origin, strength and mode of action (Roth et al. 2004). Among them, the helper component proteinase (HC-Pro) codified by Potyvirus genomes is a well known suppressor of gene silencing that prevents the accumulation of siRNAs and, as consequence, the establishment of gene silencing (Vance, 1991).

Experimental approaches to demonstrate RNA gene silencing

Even though gene silencing exists among eukariotic organisms, plants are the most widely used model system to illustrate gene silencing, because they are easy and inexpensive to handle (Fire et al. 1998; Voinnet 2005a).

Transient expression assays in plants can be performed by a procedure called agro-infiltration that makes use of the bacterial plant pathogen *Agrobacterium tumefaciens*. During infection, Agrobacterium transfers T-DNA that is part of its plasmidial DNA to the infected plant. The *Agrobacterium* strains employed for infiltration contain the plasmid carrying the sequences in study, which will be expressed in the plant tissue. The technique consists in the

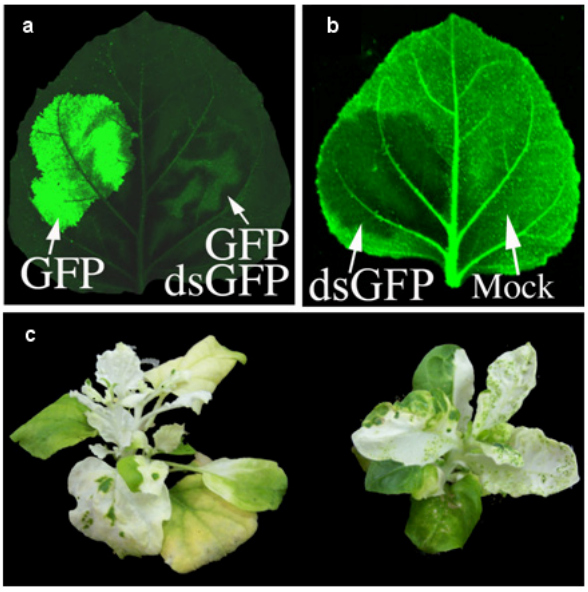


Figure 1. Triggering gene silencing.
(a) *N. benthamiana* leaf, agroinfiltrated with *Agrobacterium* bearing 35S-*gfp* (left side) and 35S-*gfp* plus 35S-ds *gfp* constructs (right side) observed under UV light.
(b) Transgenic leaf expressing GFP (line 16C) agroinfiltrated with 35S-ds *gfp* (left side) and mock inoculated observed under UV light.
(c) *N. benthamiana* (left panel) and *N. tabacum* (right panel) plants agroinfiltrated with TRV1 and TVR2 carrying a PDS sequence.

infiltration of the abaxial surface of a leaf with an *Agrobacterium* suspension using a needle-less syringe (Movie 1). One well suited plant model to this approach is *Nicotiana benthamiana*, whose leafs can be easily infiltrated and produce high levels of protein.

In order to perform gene silencing assays in plants, reporter genes are used that allow a rapid and quantitative way to distinguish if they are active or silenced. The jellyfish gene encoding for the green fluorescent protein (*gfp*) is one of the most widely used reporter genes. Its expression results in a green fluorescent emission under UV light (Stearns, 1995). Due to chlorophyll emission, wild type healthy plant tissues look bright red under UV light; therefore areas expressing GFP contrast with non agroinfiltrated areas exposed to the same light source. Other frequently reporter genes used in gene silencing assays are those implicated in the biosynthesis of plant pigments. Silencing induction of this type of genes leads to the reduction of colored compounds accumulation, a phenomenon that can be easily noticed with just bare eyes (Fofana et al. 2004). For example, phytoene desaturase protein (PDS) is a key enzyme in the carotenoid biosynthesis pathway. Silencing of the PDS gene (*pds*) results in white leaves, a phenotype called photobleaching.

This work is focused to: 1. Triggering of gene silencing; 2. Systemic gene silencing and finally 3. Gene silencing suppression. The first topic, describes experiments that illustrate the phenomenon of gene silencing of transgenes or endogenous genes, triggered by either transient expression of homologous sequences or viruses carrying them. This section is recommended to be taught in basic courses such as Genetics and Molecular Biology. The second topic illustrates systemic gene silencing showing how the silencing signal is spread over a plant. Finally the third topic exemplifies suppression of gene silencing. The second and third sections are orientated to more advanced courses in Biotechnology and Virology areas. These experiments were recently used as experimental practice for a Molecular Phytopathology course taught at the University of Buenos Aires, Argentina.

Objectives

The aim of this work is to provide simple and inexpensive assays to teach gene silencing using plants as model systems. These experiments provide a nice, colorful and conclusive image of plant gene silencing. Hopefully, it will help students to understand this important phenomenon and to unravel the importance of gene silencing (and its suppression) as a key gene regulation mechanism and as a molecular and biotechnological tool.

MATERIALS AND METHODS

Plant materials

Transgenic and non-transgenic plants were grown under standard greenhouse conditions or maintained in growth chambers at 24 to 26ºC, under a 14 hrs light / 10 hrs dark cycle. The following transgenic lines were used: *Nicotiana tabacum* silenced for magnesium chelatase (Dr. Christopher Taylor from the Donald Danforth Plant Science Center, USA) and *N. benthamiana* expressing GFP (line 16C; Dr. David Baulcome from the John Innes Center, UK) (Brigneti et al. 1998).

Agrobacterium infiltration procedure

Agrobacterium tumefaciens strain GV3101 carrying the different constructs were plated in LB-Agar medium with an appropriate antibiotic (Table 1) and maintained at 28ºC for 2 days (at least 36 hrs). One fresh colony was used to inoculate 3 ml of LB with the proper antibiotic (Table 1) and grown overnight (at least 16 hrs) at 28ºC. 0.5 ml of the overnight culture was used to inoculate 20 ml of LB media containing the proper antibiotics (Table 1) and acetosyringone (20 µM final) and grew overnight. The bacteria were then centrifuged at 5000 rpm for 10 min. The pellet was resuspended in agroinfiltration solution (10 mM $MgCl_2$; 10 mM MES and 100 µM acetosyringone) up to the desired optic density (OD 600) (Table 1). The bacteria solution was left at room temperature for 2 to 4 hrs before agroinfiltration. The infiltration was performed as shown in Movie 1. Figure 4 show the correct age and sizes of the plants to be used on each different assay. In the case where

Figure 2. Systemic silencing progression.
(a) Transgenic plants expressing GFP (line 16C) observed under UV light.
(b) Transgenic leaves of line 16C agroinfiltrated with 35S-ds *gfp* after 1 week. The black dashed line marks the agroinfiltrated zone.
(c) and (d) GFP plants agroinfiltrated with 35S-ds *gfp* after 2 and 3 weeks respectively, the GFP signal is reduced while the gene silencing signal is spread.
(e) Non transgenic plant under the UV light.

the TRV system was used; equal volume of the strains carrying TRV1 and TRV2-PDS cultures were mixed to inoculate the plants.

DNA constructs

The following DNA constructs were used: 35S-*gfp*, 35S-ds *gfp* and 35S-HC-Pro (Dr. David Baulcome from the John Innes Centre, UK) and TRV constructs pTRVRNA1 and pTRVRNA2-nbPDS (Professor Savithramma Dinesh-Kumar from Yale University, USA), Table 2.

GFP observation

Plants expressing GFP were observed under a strong UV lamp (Black ray B-100AP 100 Watts, UVP®, USA) in a completely dark room. To use the UV lamp it is required to wear UV protection goggles and avoid exposure of the naked skin to the UV light. Whole plants pictures for this work were taken using a Kodak digital camera using a UV filter. The GFP florescence of the agroinfiltrated leaves for this work was documented using a Typhoon Scanner (Amersham Biosciences, UK).

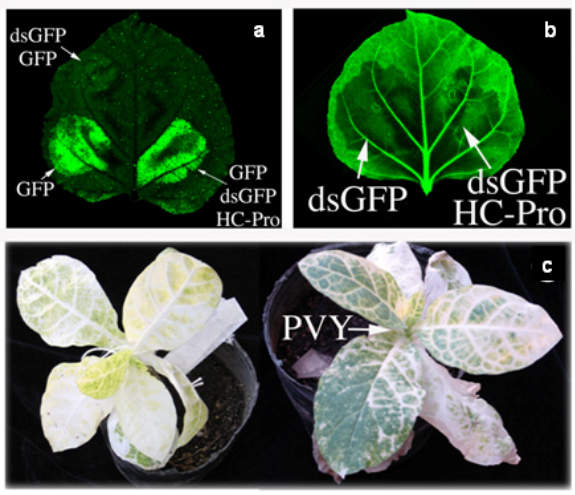


Figure 3. Suppression of gene silencing.
(a) *N. benthamiana* leaf observed under UV light, agroinfiltrated with strains bearing 35S-*gfp*; 35S-*gfp* + 35S-ds *gfp* or 35S-*gfp* + 35S-ds *gfp* + 35S-HC-Pro constructs.
(b) Transgenic GFP leaf (line 16C) agroinfiltrated with 35S-ds *gfp* or 35S-ds *gfp* + 35S-HC-Pro.
(c) *N. tabacum* silenced for endogenous *pds* showing the photobleaching phenotype (left panel) and the same plant infected with PVY after 2 weeks (right panel). The arrow points at the initially infected leaf.

RESULTS

Triggering gene silencing

Here we describe three experimental approaches to evidence RNA silencing. In the first approach gene silencing is demonstrated after transient expression of reporter genes in non-transgenic plants. In the second, silencing is triggered against a reporter gene stably integrated into a transgenic plant. The third and last approach involves the triggering of RNA silencing against endogenous genes using viral vectors.

Gene silencing of transiently expressed sequences. Infiltration of non-transgenic *Nicotiana benthamiana* leaves with a strain of *Agrobacterium tumefaciens* carrying a *gfp* gene construct (35S-*gfp*) results in transient GFP expression, observed as a green fluorescence under UV illumination (Figure 1a) (Llave et al. 2000; Voinnet et al. 2000; Dunoyer et al. 2002; Hamilton et al. 2002; Takeda et al. 2002; Bucher et al. 2003). In contrast, co-infiltration of agrobacteria carrying a 35S-*gfp* construct together with a strain carrying an inverted repeat of *gfp* construct (35S-ds *gfp*) does not produce any green fluorescence. This phenomenon is a consequence of gene silencing triggered by 35-ds *gfp* resulting in a drastic reduction of the GFP level (Figure 1a). In this experiment, siRNAs are produced from the 35S-ds *gfp* construct; those siRNAs (homologous to the *gfp* sequence) guide the recognition by RISC of GFP mRNAs expressed from the 35S-*gfp* construct that in turn leads to its degradation (Slide 2 Power Point Presentation 1). Therefore, no fluorescent signal is detected and the plants look red due to normal chlorophyll emission, the same as mock-infiltrated controls under UV light.

Table 1. Schedule for all the silencing experiments.

Days			
1	Plate the agrobacterium in LB-Agar plate with appropriate antibiotic		
	1a, 1b, 2, 3a, 3b	35-ds GFP	Kan50mg/ml; Genta 40mg/ml and Rif 100mg/ml
	1a, 3a	35-GFP	Kan50mg/ml; Genta 40mg/ml and Rif 100mg/ml
	3a, 3b	35-Hc-PRO	Kan50mg/ml; Genta 40mg/ml and Rif 100mg/ml
	1c	TRV1	Kan50mg/ml; Genta 40mg/ml and Rif 100mg/ml
	1c	TRV2	Kan50mg/ml; Genta 40mg/ml and Rif 100mg/ml
	At this time the plants should have the proper size to be agroinfiltrated (Figure 4)		
2	Pick a colony up and inoculate 5 ml with the appropriate antibiotic Incubate at 28ºC with shaker		
3	Used 0.5 ml of the overnight culture to inoculate 20 ml of LB media Correct antibiotic and Acetosyringone (20 mM final) Incubate at 28ºC with shake		
4	Centrifuge the bacteria (5000 rpm for 10 min) Resusped in groinfiltration solution 10 mM MgC12; 10 mM MES and 100 µM Acetosyringone Quantify the OD (600) of the bacterias and make the appropriate dilution		
	1a, 1b, 2 1a 1a 1c 1c	35-ds GFP 35-GFP 35-Hc-PRO TRV1 TRV2	1 OD final 0.5 OD final 0.5 OD final 1 OD final 1 OD final
	Leave the bacteria solution at room temperature for 2 to 4 hrs Infiltrate the underside leaf with a 2 or 10 ml syringe. See Movie 1 Maintain the plants in growth chambers at NO more than 28ºC, 14 hrs light cycle		
6 to 10	Silencing efects should be seen 2 to 10 days post the infiltrations days.		
	1a, 3a 1b, 3b 1c, 2 3c	2 days should be enough but 4 or 5 days in better 5 days should be enough 1 week should be enough see light effects and 2 to 4 weeks to see strong ones 2 weeks should be enough	

1a. Gene silencing of transiently expressed sequences.
1b. Triggering gene silencing of established transgene by transient expression.
1c. Virus-induced gene silencing (VIGS).
2. Systemic gene silencing.
3a. Suppressing gene silencing of transiently expressed sequences.
3b. Silencing of transgenes or endogenous genes cannot be established in the presence of silencing suppressor.

Triggering gene silencing of stable transgene by transient expression. It is also possible to trigger PTGS of endogenous genes or stable transgenes through transient expression of homologous sequences. A *N. benthamiana* transgenic line expressing GFP (line 16C) displays a strong green fluorescent signal under UV light (Ruiz et al. 1998). When leaves of seven weeks old N.b. 16C plants are agroinfiltrated with strains carrying 35S-*gfp* or 35S-ds *gfp* constructs, the agro-infiltrated tissues change to red color under UV light one week post infiltration. The loss of the

green fluorescent signal indicates reduction of transgenic GFP expression level or, in other words, specific GFP silencing (Figure 1b). The same strategy can be used to induce the silencing of any endogenous gene in *N. benthamiana* as long as its sequence is known and the constructs expressing the desired sequence are used.

Virus-induced gene silencing (VIGS). The most commonly used viruses to induce RNA silencing are *Tobacco rattle virus* (TRV) and *Potato virus X* (PVX) (Lu et al. 2003; Burch-Smith et al. 2004; Robertson, 2004). Both produce mild symptoms in *Solanaceae* plants species that do not interfere with the outcome phenotype. The infection is carried out by agro-infiltrating plant tissue with a plasmid vector containing an infective copy of the viral genome. The viral genomes are genetically modified to allow the expression of a foreign sequence from a viral promoter. PVX and TRV have positive single stranded RNA genomes, therefore the transcription of one copy of the viral genome from the plasmid vector results in the generation of one infective virus that can perfectly replicate and infect the whole plant. TRV has a bipartite genome formed by RNA1 and RNA2 molecules. RNA1 contains genes essential for virus replication, including the replicase and movement protein. It can replicate and move systemically without the presence of RNA2. RNA2 codes for the coat protein and for two non-structural proteins that are required for virus spread from plant to plant. Liu et al. (2002) engineered two constructs harboring the RNA1 or RNA2 under the 35S promoter called TRV1 and TRV2 respectively (Wesley et al. 2001). TRV2 was further engineered and the two non structural genes were removed and a multiple cloning site was added; therefore any sequence can be easily cloned into the virus genome and then expressed together with the virus replication (Ratcliff et al. 2001).

Inoculation of *N. benthamiana* plants with a modified TRV (TRV1 and TRV2) carrying host-derived inserts, produces a reduction of the expression of the endogenous homologous gene 7 to 15 days post infection. Figure 1c, shows *N. benthamiana* and *N. tabacum* plants infected by means of agro-infiltration using the infectious TRV constructs, carrying a modified TRV2, harboring a portion of 400 bp sequence from *pds* gene show a photobleaching phenotype The leafs look almost completely white due to the silencing of the endogenous gene (photobleaching phenotype).

As a conclusion for this section, gene silencing is a natural phenomenon in plants that is triggered by dsRNAs. This dsRNA molecules can be originated by the expression of constructs bearing sense and antisense sequences (which transcription give rise to dsRNAs) and by viral replication. Any of these approaches can be used to study the effect of the functional knock out (gene silencing) of any desired gene.

Systemic gene silencing

As mentioned in the introduction, the silencing signal spreads over the entire plant. Here we illustrate the spread of the gene silencing signal from the agroinfiltrated zone to the entire plant giving rise to systemic gene silencing.

When a lower leaf of a N.b. 16C plant that expresses GFP under the 35Ss promoter is agroinfiltrated with a strain carrying a 35S-ds *gfp* construct, the green fluorescent signal observed under UV light is lost in the agroinfiltrated leaf after 7 days. The strong green fluorescent signal of the rest of the plant leaves (systemic) gradually starts to decrease and, after 3 to 4 weeks, the whole N.b. 16C plant looks red as a non transgenic plant observed under the UV light. As shown in Figure 2; silencing of GFP is initiated in the infiltrated area (Figure 2b) and is followed by the spreading of GFP-specific siRNAs through the entire plant, thus producing systemic GFP silencing and the consequent reduction of the fluorescent signal (Figure 2c and Figure 2d).

As a conclusion, gene silencing initiates where it is triggered, then spreads along the entire leaf and finally moves from one leaf to the others using the vascular tissues to ultimately reach the entire plant. It was demonstrated that the cell-to-cell mobile silencing signal is a 21 nt siRNA molecule that is spread using a “relay” process where it is

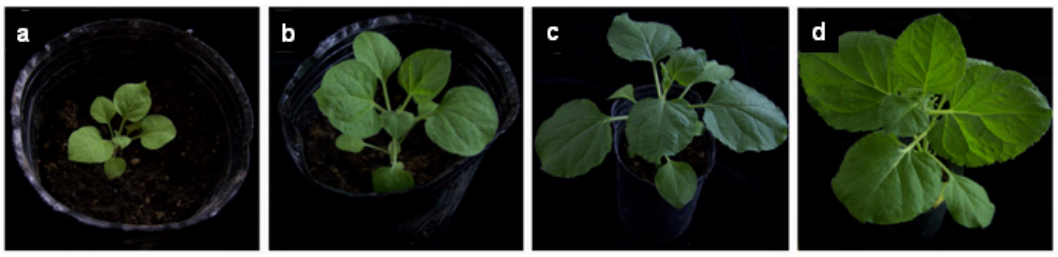


Figure 4. Plant material to use on the assays.
(a) and (b) Correct size of *N. benthamiana* plants for systemic gene silencing experiments.
(c) and (d) Correct size of *N. benthamiana* plants for all the silencing experiments except for systemic spread.

re-amplified by a cellular RNA-dependent RNA polymerase every 10-15 cells (Himber et al. 2003; Yoo et al. 2004; Voinnet, 2005b).

Gene silencing suppression

As mentioned before, viruses have evolved to encode for proteins that suppress gene silencing. In this section we will exemplify gene silencing suppression using the same experimental approaches as before but adding the expression of the well characterized potyviral suppressor, HC-Pro. Finally, we will show how a virus can suppress an established gene silencing during a natural infection process.

Suppressing gene silencing of transiently expressed sequences. As shown in Figure 1a, transient expression of 35S-*gfp* gives rise to a bright green area in a non-transgenic plant under the UV light (Figure 3, lower left side). In contrast, when 35S-*gfp* and 35S-ds *gfp* are co-expressed, no florescence is detected because of GFP silencing triggered by the ds *gfp* construct (Figure 1a and Figure 3a, upper right side). When a strong gene silencing suppressor as HC-Pro, is co-expressed with constructs 35S-*gfp* and 35S-ds *gfp*, the infiltrated zone remains bright green (Figure 3a, lower right side of the leaf). This is due to the inhibition of the silencing machinery caused by HC-Pro. Specifically HC-Pro inhibits an intermediate step of RNA silencing via binding to siRNAs. In addition it inhibits the siRNA-initiated RISC assembly pathway by preventing RNA silencing initiator complex formation. (Lakatos et al. 2006).

Silencing of transgenes or endogenous genes cannot be established in the presence of a silencing suppressor. Gene silencing suppressors can also inhibit silencing of transgenes or endogenous genes. As shown in Figure 1b, when leaves of the N.b. 16C plants were agro-infiltrated with a strain carrying the construct 35S-ds *gfp*, the strong green fluorescent signal disappeared under UV light due to GFP silencing (Figure 1b and Figure 3b left side of the leaf). However, if 35S-ds *gfp* and 35-HC-Pro are co-agroinfiltrated, the fluorescent signal does not disappear and the area does not look dark under the UV light (Figure 3b, right side of the leaf). This is due to the inhibition of gene silencing caused by HC-Pro.

Suppression of gene silencing mediated by virus infection. Gene silencing can be permanently triggered in transgenic plants by designing and using a transgene containing a sense and antisense copy of the gene to be silenced in such a way that a dsRNA is produced (Wesley et al. 2001). *Nicotiana tabacum* and *Nicotiana benthamiana* silenced for the magnesium chelatase gene show a characteristic photobleaching phenotype (Figure 3c, left plant) (Fofana et al. 2004). However, the photobleaching phenotype is markedly reduced and the green pigmentation restored (Figure 3c, right plant) when the silenced plants are infected with PVY. This indicates that the replication of PVY was able to suppress the established magnesium chelatase silencing. As we previously mentioned PVY carries a gene encoding for a strong silencing suppressor called HC-Pro. Therefore, proteins able to suppress gene silencing can be easily detected through this experimental system and so any viruses that may carry them. Most of the viruses have some sort of silencing suppressor system as an evolutionary counter-response to the antiviral activity of the plant gene silencing defense.

CONCLUDING REMARKS

RNA silencing has become a hot topic of biological research in the last few years. Understanding gene silencing is highly relevant to comprehend the interaction between virus and their hosts and, in a wider view, to comprehend the relationships between foreign RNAs (including retrotransposons, viroids, etc.) and eukaryotic cells. Moreover, gene silencing reveals an entirely new level of post-transcriptional gene regulation. Furthermore it became an extremely useful technique for molecular biology and a very powerful biotechnological tool. Here, we performed a detailed description of the most common assays related to gene silencing that will provide simple and inexpensive tools to help to the understanding and teaching of RNA

Table 2. DNA constructs table.

Original vectors	Referred as	Origin	Institution
pBIN61-GFP	35S-*gfp*	Baulcombe, D.	John Innes Centre, UK
pBICdsGFP	35S-ds gfp and	Okuno, T.	Kyoto University; Japan
pBIN61-HC-Pro	35S-HC-Pro	Baulcombe, D.	John Innes Centre, UK
pTRV-RNA1	pTRVRNA1	Dinesh-Kumar, S.	Yale University, USA
pTRV2-nbPDS	pTRVRNA2-nbPDS	Dinesh-Kumar, S.	Yale University, USA

silencing. We consider the topic is of vital importance for the students interested in biotechnology and plant pathology.

ACKNOWLEDGMENTS

We are grateful to Dr. David Baulcombe/Sainsbury Laboratory/The Gatsby Charitable Foundation. (John Innes Centre) for the GFP 16C plant line, 35S-gfp, and 35S-HC-Pro constructs. We thank Kazuyuki Mise for providing 35S-dsgfp (Laboratory of Plant Pathology, Graduate School of Agriculture, Kyoto University, Kyoto, Japan). We thank Professor Savithramma Dinesh-Kumar (Yale University) for providing the TRV vectors (TRV1 and TRV2nbPDS). We also thank Dr. Christopher Taylor (Donald Danforth Plant Science Center) for kindly providing the transgenic plants in which the magnesium chelatase gene is silenced. We thank Guillermo Maroniche for help with the photographs and videotaping.

REFERENCES

ANANDALAKSHMI, Radhamani; PRUSS, Gail J.; GE, Xin; MARATHE, Rajendra; MALLORY, Allison C.; SMITH, Trenton H. and VANCE, Vicki B. A viral suppressor of gene silencing in plants. *Proceedings of the National Academy of Sciences of the United States of America*, October 1998, vol. 95, no. 22, p. 13079-13084.

ANANDALAKSHMI, Radhamani; MARATHE, Rajendra; GE, Xin; HERR, John M. Jr.; MAU, Christopher; MALLORY, Allison; PRUSS, Gail; BOWMAN, Lewis and VANCE, Vicki B. A calmodulin-related protein that suppresses posttranscriptional gene silencing in plants. *Science*, October 2000, vol. 290, no. 5489, p. 142-144.

ARAVIN, Alexei A.; NAUMOVA, Natalia M.; TULIN, Alexei V.; VAGIN, Vasilii V.; ROZOVSKY, Yakov M. and GVOZDEV, Vladimir A. Double-stranded RNA-mediated silencing of genomic tandem repeats and transposable elements in the *D. melanogaster germline*. *Current Biology*, July 2001, vol. 11, no. 13, p. 1017-1027.

BAULCOMBE, David. RNA silencing in plants. *Nature*, September 2004, vol. 431, no. 7006, p. 356-363.

BERNSTEIN, Emily; CAUDY, Amy A.; HAMMOND, Scott M. and HANNON, Gregory J. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature*, January 2001, vol. 409, no. 6818, p. 363-366.

BRIGNETI, Gianinna; VOINNET, Olivier; LI, Wan-Xiang; JI, Liang-Hui; DING, Shou-Wei and BAULCOMBE, David C. Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *The EMBO Journal*, November 1998, vol. 17, no. 22, p. 6739-6746.

BUCHER, Etienne; SIJEN, Titia; DE HAAN, Peter; GOLDBACH, Rob and PRINS, Marcel. Negative-strand tospoviruses and tenuiviruses carry a gene for a suppressor of gene silencing at analogous genomic positions. *Journal of Virology*, January 2003, vol. 77, no. 2, p. 1329-1336.

BURCH-SMITH, Tessa M.; ANDERSON, Jeffrey C.; MARTIN, Gregory B. and DINESH-KUMAR, Savithramma P. Applications and advantages of virus-induced gene silencing for gene function studies in plants. *The Plant Journal*, September 2004, vol. 39, no. 5, p. 734-746.

DUNOYER, Patrice; PFEFFER, Sébastien; FRITSCH, Christiane; HEMMER, Odile; VOINNET, Oliver and RICHARDS, Kenneth E. Identification, subcellular localization and some properties of a cysteine-rich suppressor of gene silencing encoded by peanut clump virus. *The Plant Journal*, March 2002, vol. 29, no. 5, p. 555-567.

DUNOYER, Patrice; HIMBER, Christophe and VOINNET, Olivier. Induction, suppression and requirement of RNA silencing pathways in virulent *Agrobacterium tumefaciens* infections. *Nature Genetics*, February 2006, vol. 38, no. 2, p. 258-263.

FIRE, Andrew; XU, Siquin; MONTGOMERY, Mary K.; KOSTAS, Steven A.; DRIVER, Samuel E. and MELLO, Craig C. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, February 1998, vol. 391, no. 6669, p. 806-811.

FOFANA, Ismael B.; SANGARE, Abdourahamane; COLLIER, Ray; TAYLOR, Christopher and FAUQUET, Claude M. A geminivirus-induced gene silencing system for gene function validation in cassava. *Plant Molecular Biology*, November 2004, vol. 56, no. 4, p. 613-624.

HAMILTON, Andrew; VOINNET, Olivier; CHAPPELL, Louise and BAULCOMBE, David. Two classes of short interfering RNA in RNA silencing. *The EMBO Journal*, September 2002, vol. 21, no. 17, p. 4671-4679.

HAMMOND, Scott M.; BERNSTEIN, Emily; BEACH, David and HANNON, Gregory J. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature*, March 2000, vol. 404, no. 6775, p. 293-296.

HIMBER, Christophe; DUNOYER, Patrice; MOISSIARD, Guillaume; RITZENTHALER, Christophe and VOINNET, Olivier. Transitivity-dependent and -independent cell-to-cell movement of RNA silencing. *The EMBO Journal*, September 2003, vol. 22, no. 17, p. 4523-4533.

LAKATOS, Lóránt; CSORBA, Tibor; PANTALEO, Vitantonio; CHAPMAN, Elisabeth J.; CARRINGTON, James C.; LIU, Yu-Ping; DOLJA, Valerian V.; FERNANDEZ CALVINO, Lourdes; LOPEZ-MOYA, Juan José and BURGYAN, József. Small RNA binding is a common strategy to suppress RNA silencing by several

viral suppressors. *The EMBO Journal*, June 2006, vol. 25, no. 12, p. 2768-2780.

LI, Hongwei; LI, Wan Xiang and DING, Shou Wei. Induction and suppression of RNA silencing by an animal virus. *Science*, May 2002, vol. 296, no. 5571, p. 1319-1321.

LIU, Yule; SCHIFF, Michael and DINESH-KUMAR, S.P. Virus-induced gene silencing in tomato. *The Plant Journal*, September 2002, vol. 31, no. 6, p. 777-786.

LU, Rui; MARTIN-HERNANDEZ, Ana Montserrat; PEART, Jack R.; MALCUIT, Isabelle and BAULCOMBE, David C. Virus-induced gene silencing in plants. *Methods*, August 2003, vol. 30, no. 4, p. 296-303.

LUCY, Andrew P.; GUO, Hui-Shan; LI, Wan-Xiang and DING, Shou-Wei. Suppression of post-transcriptional gene silencing by a plant viral protein localized in the nucleus. *The EMBO Journal*, April 2000, vol. 19, no. 7, p. 1672-1680.

LLAVE, Cesar; KASSCHAU, Kristin D. and CARRINGTON, James C. Virus-encoded suppressor of posttranscriptional gene silencing targets a maintenance step in the silencing pathway. *Proceedings of the National Academy of Sciences of the United States of America*, November 2000, vol. 97, no. 24, p. 13401-13406.

MALLORY, Allison C.; ELY, Lara; SMITH, Trent H.; MARATHE, Rajendra; ANANDALAKSHMI, Radhamani; FAGARD, Mathilde; VAUCHERET, Herve; PRUSS, Gail; BOWMAN, Lewis and VANCE, Vicki B. HC-Pro suppression of transgene silencing eliminates the small RNAs but not transgene methylation or the mobile signal. *The Plant Cell*, March 2001, vol. 13, no. 3, p. 571-583.

RATCLIFF, Frank; MARTIN-HERNANDEZ, Ana Montserrat and BAULCOMBE, David C. Technical Advance. Tobacco rattle virus as a vector for analysis of gene function by silencing. *The Plant Journal*, January 2001, vol. 25, no. 2, p. 237-245.

ROBERTSON, Dominique. VIGS vectors for gene silencing: many targets, many tools. *Annual Review of Plant Biology*, June 2004, vol. 55, p. 495-519.

ROTH, Braden M.; PRUSS, Gail J. and VANCE, Vicki B. Plant viral suppressors of RNA silencing. *Virus Research*, June 2004, vol. 102, no. 1, p. 97-108.

RUIZ, M. Teresa; VOINNET, Olivier and BAULCOMBE, David C. Initiation and maintenance of virus-induced gene silencing. *The Plant Cell*, June 1998, vol. 10, no. 6, p. 937-946.

STEARNS, Tim. Green fluorescent protein: The green revolution. *Current Biology*, March 1995, vol. 5, no. 3, p. 262-264.

TAKEDA, Atsushi; SUGIYAMA, Kazuhiko; NAGANO, Hideaki; MORI, Masashi; KAIDO, Masanori; MISE, Kazuyuki; TSUDA, Shinya and OKUNO, Tetsuro. Identification of a novel RNA silencing suppressor, NSs protein of Tomato spotted wilt virus. *FEBS Letters*, December 2002, vol. 532, no. 1-2, p. 75-79.

TANG, Guiliang; REINHART, Brenda J.; BARTEL, David P. and ZAMORE, Phillip D. A biochemical framework for RNA silencing in plants. *Genes and Development*, January 2003, vol. 17, no. 1, p. 49-63.

VANCE, Vicki Bowman. Replication of potato virus X RNA is altered in coinfections with potato virus Y. *Virology*, June 1991, vol. 182, no. 2, p. 486-494.

VOINNET, Olivier; LEDERER, Carsten and BAULCOMBE, David C. A viral movement protein prevents spread of the gene silencing signal in *Nicotiana benthamiana*. *Cell*, September 2000, vol. 103, no. 1, p. 157-167.

VOINNET, Olivier. Induction and suppression of RNA silencing: insights from viral infections. *Nature Reviews Genetics*, March 2005a, vol. 6, no. 3, p. 206-220.

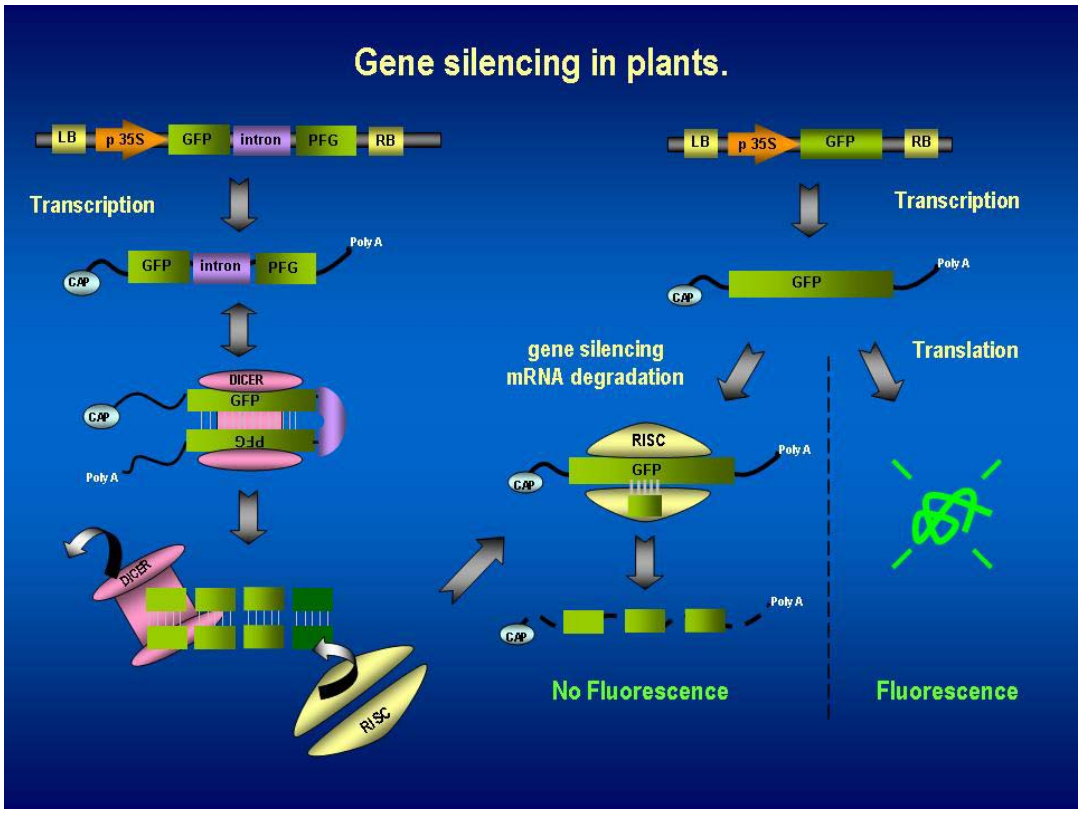
VOINNET, Olivier. Non-cell autonomous RNA silencing. *FEBS Letters*, October 2005b, vol. 579, no. 26, p. 5858-5871.

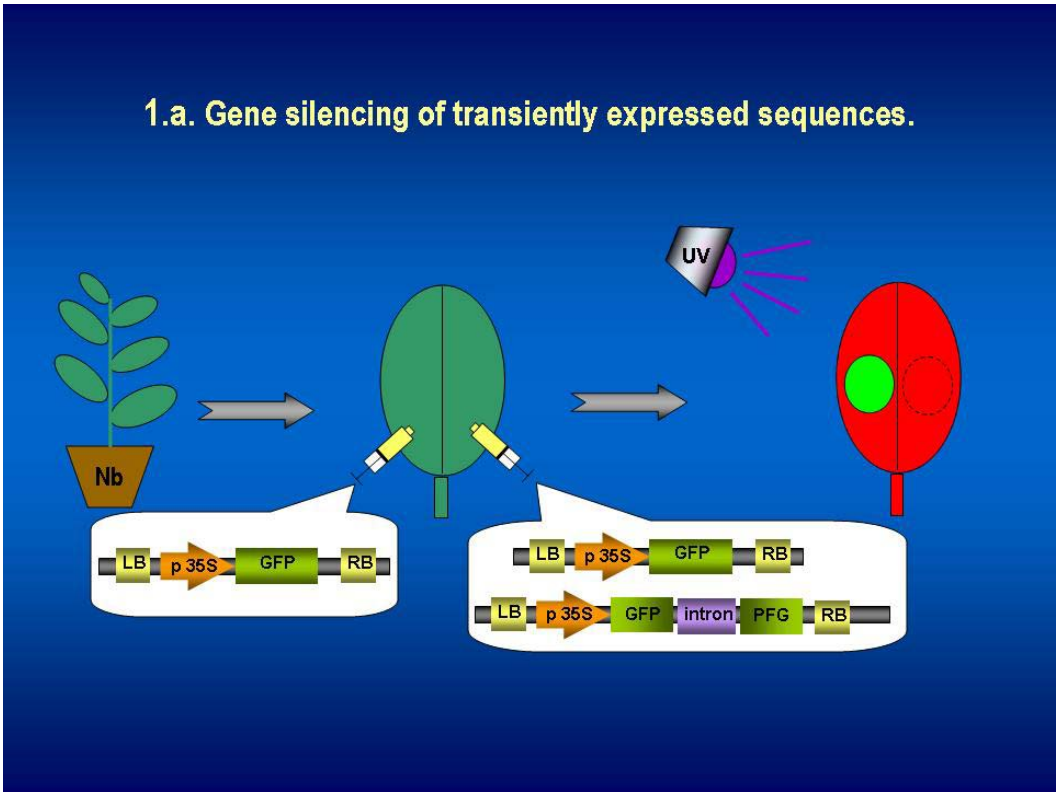
WESLEY, S. Varsha; HELLIWELL, Christopher A.; SMITH, Neil A.; WANG, Ming Bo; ROUSE, Dean T.; LIU, Qing; GOODING, Paul S.; SINGH, Surinder P.; ABBOTT, David; STOUTJESDIJK, Peter A.; ROBINSON, Simon P.; GLEAVE, Andrew P.; GREEN, Allan G. and WATERHOUSE, Peter M. Construct design for efficient, effective and high-throughput gene silencing in plants. *The Plant Journal*, September 2001, vol. 27, no. 6, p. 581-590.

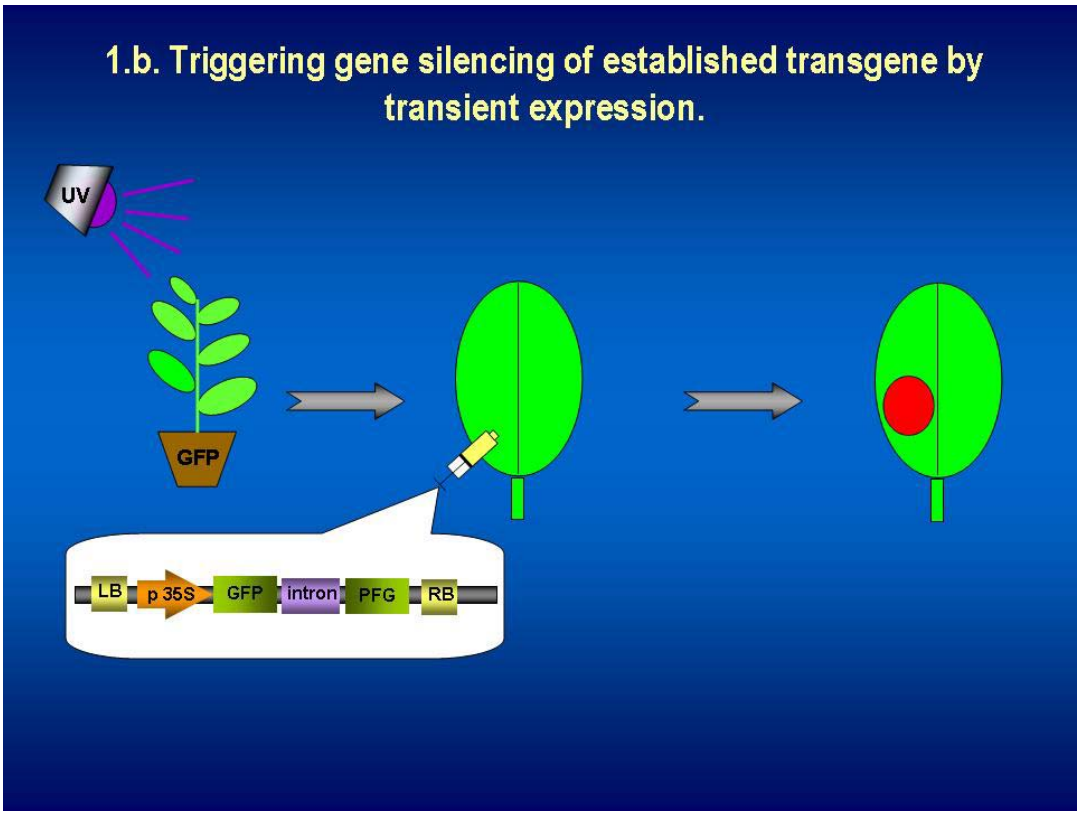
YOO, Byung-Chun; KRAGLER, Friedrich; VARKONYI-GASIC, Erika; HAYWOOD, Valerie; ARCHER-EVANS, Sarah; LEE, Young Moo; LOUGH, Tony J. and LUCAS, William J. A systemic small RNA signaling system in plants. *The Plant Cell*, August 2004, vol. 16, no. 8, p. 1979-2000.

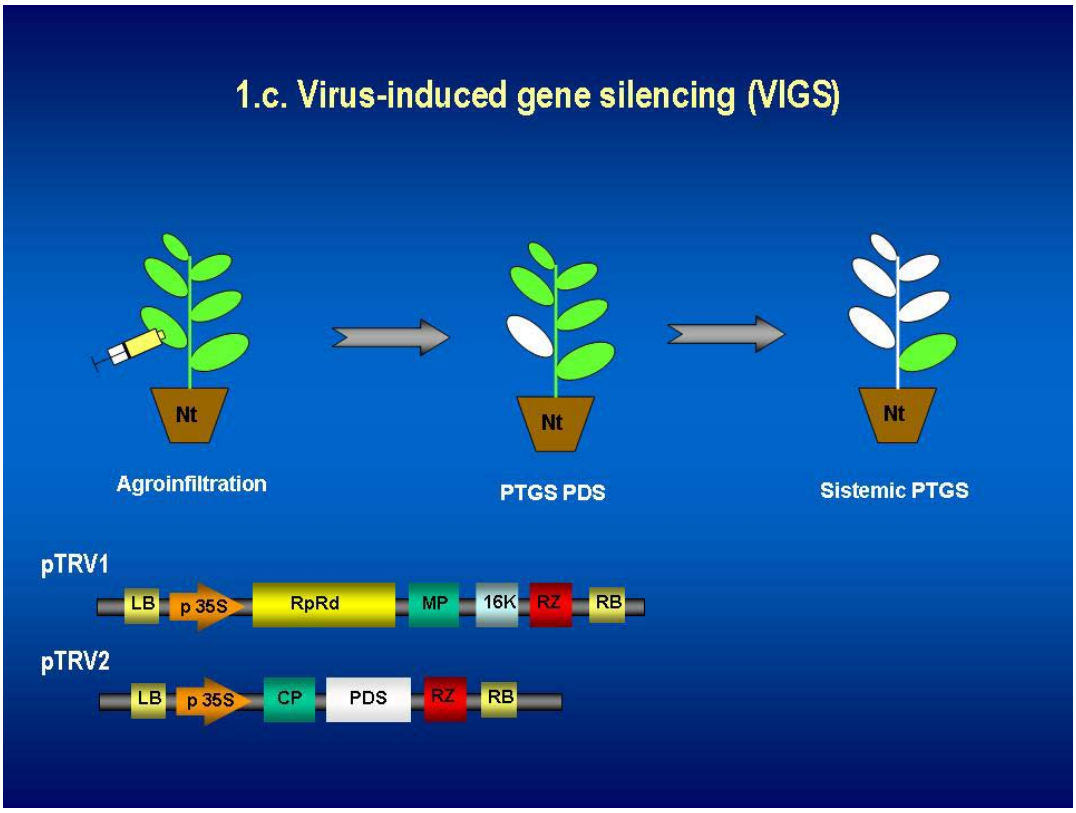
ZHOU, Z. Sh.; DELL'ORCO, M.; SALDARELLI, P.; TURTURO, C.; MINAFRA, A. and MARTELLI, G.P. Identification of an RNA-silencing suppressor in the genome of *Grapevine virus A*. *Journal of General Virology*, August 2006, vol. 87, no. 8, p. 2387-2395.

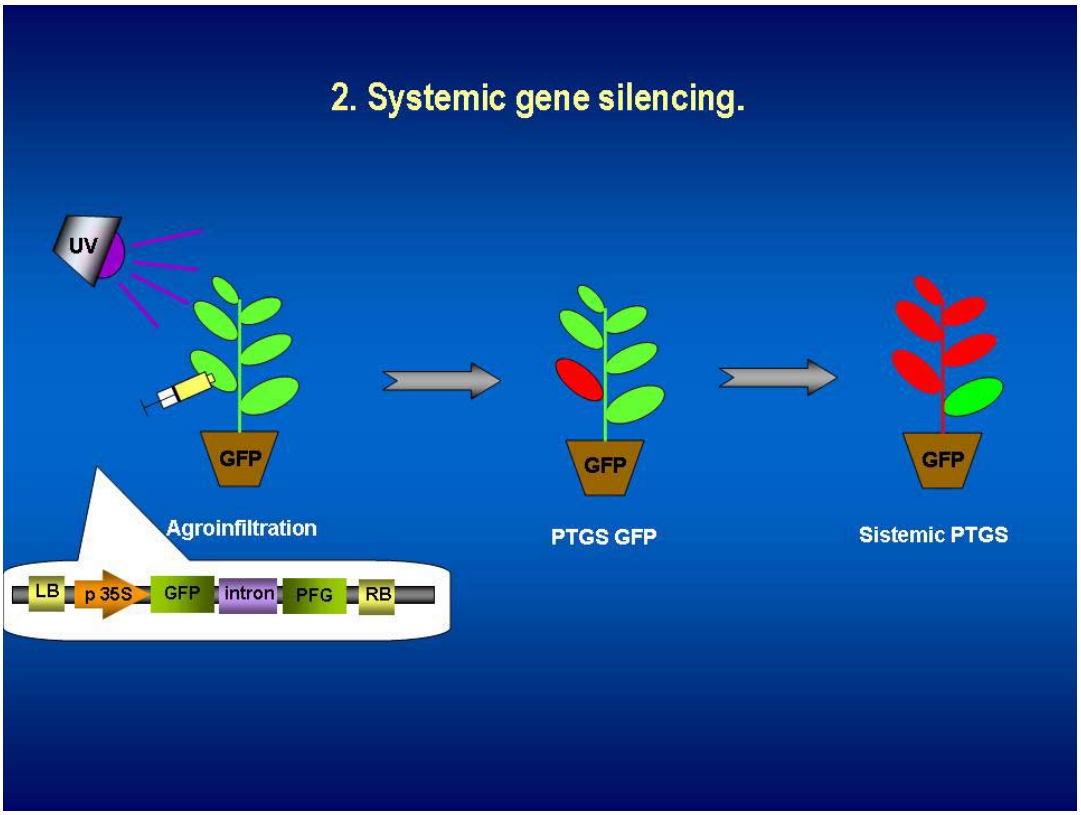
POWER POINT PRESENTATION

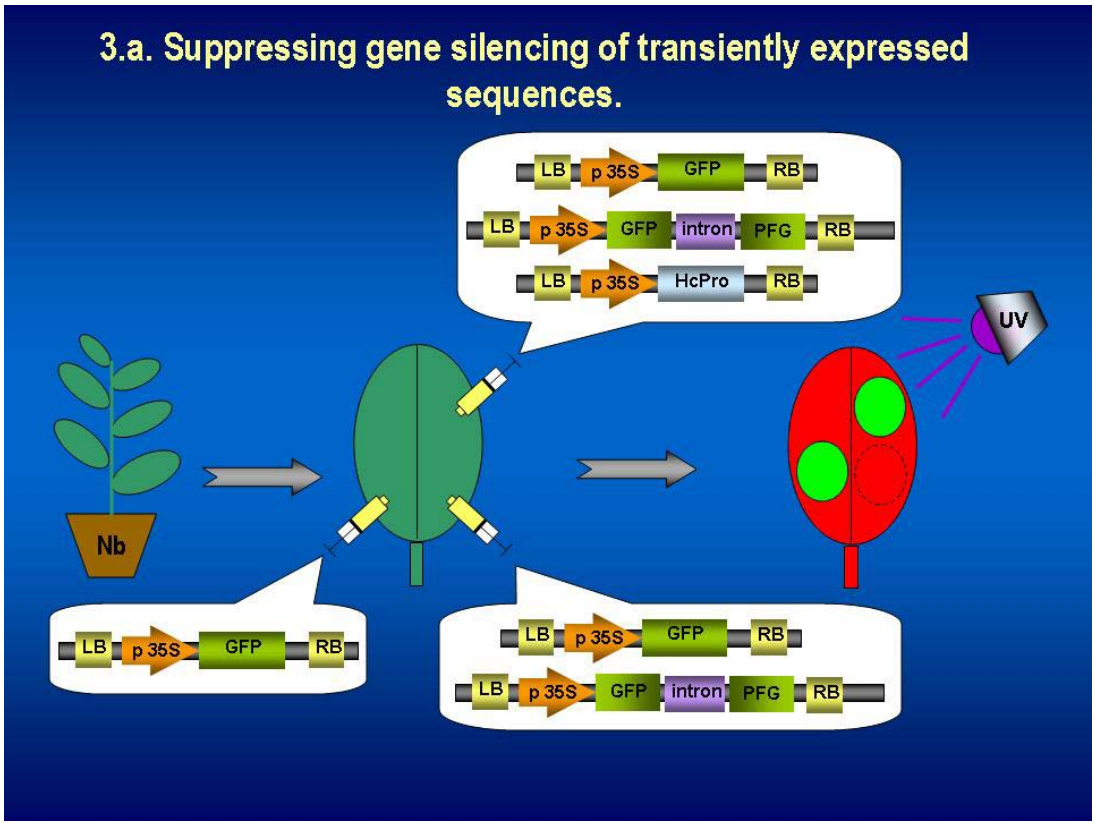


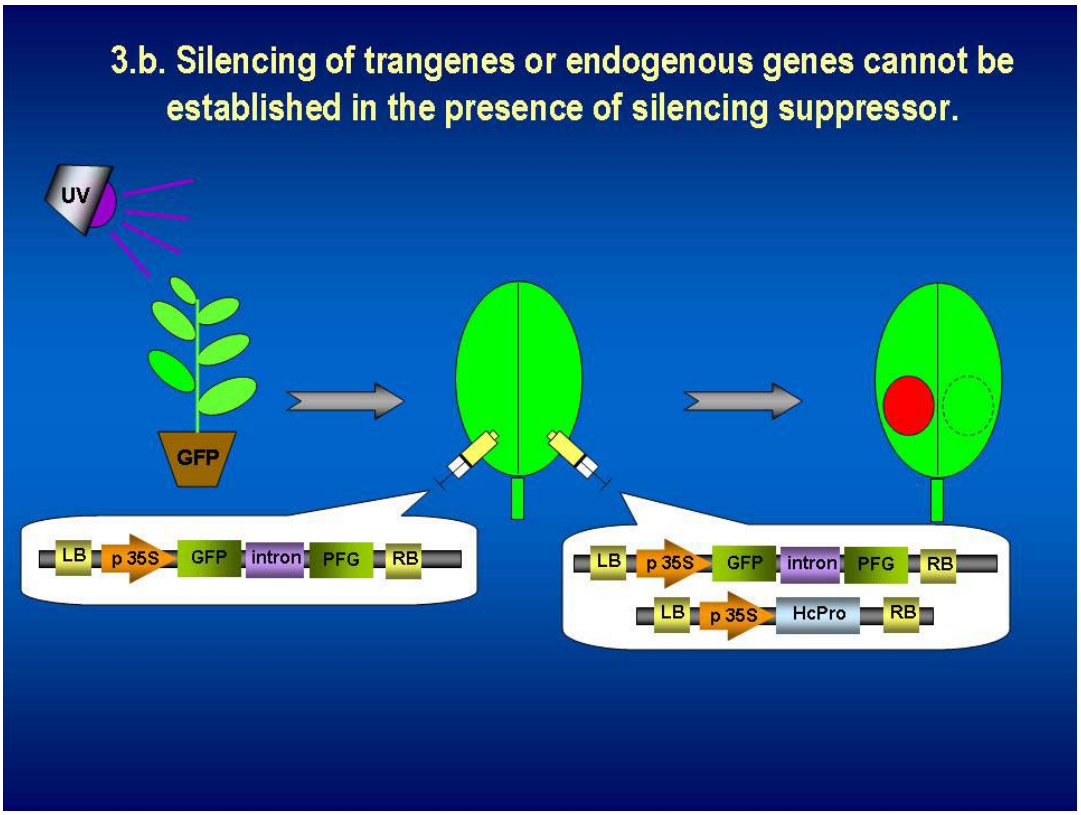


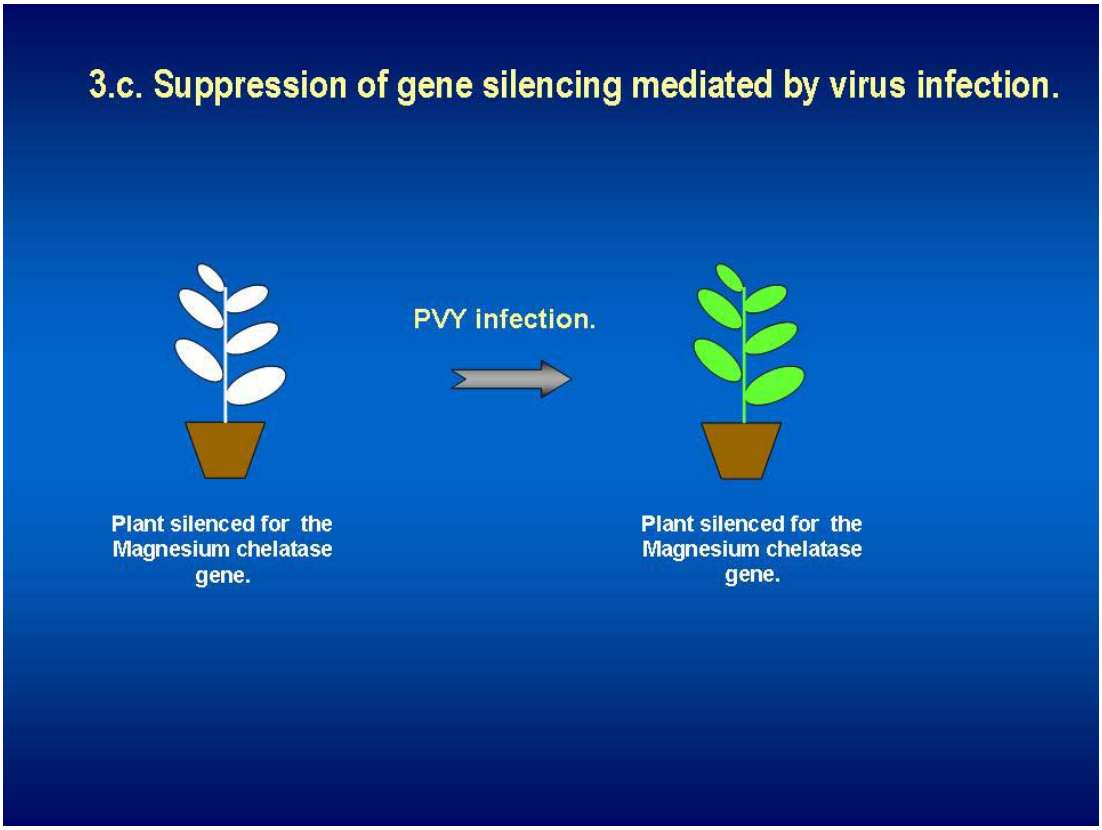

1.b. Triggering gene silencing of established transgene by transient expression.
UV
GFP
LB
p 35S
GFP
intron
PFG
RB


1.c. Virus-induced gene silencing (VIGS)
Nt
Nt
Nt
Agroinfiltration
PTGS PDS
Sistemic PTGS
pTRV1
LB
p 35S
RpRd
MP
16K
RZ
RB
pTRV2
LB
p 35S
CP
PDS
RZ
RB


2. Systemic gene silencing.
UV
GFP
Agroinfiltration
LB
p 35S
GFP
intron
PFG
RB
GFP
PTGS GFP
GFP
Sistemic PTGS


3.a. Suppressing gene silencing of transiently expressed sequences.
LB
p 35S
GFP
RB
LB
p 35S
GFP
intron
PFG
RB
LB
p 35S
HcPro
RB
UV
Nb
LB
p 35S
GFP
RB
LB
p 35S
GFP
RB
LB
p 35S
GFP
intron
PFG
RB


3.b. Silencing of trangenes or endogenous genes cannot be established in the presence of silencing suppressor.
UV
GFP
LB
p 35S
GFP
intron
PFG
RB
LB
p 35S
GFP
intron
PFG
RB
LB
p 35S
HcPro
RB


3.c. Suppression of gene silencing mediated by virus infection.
PVY infection.
Plant silenced for the Magnesium chelatase gene.
Plant silenced for the Magnesium chelatase gene.