Gene silencing induced by RNA1 16K gene mutants of Tobacco rattle virus on *Nicotiana benthamiana*

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Tiivistelmä — Referat — Abstract

Virus-induced gene silencing (VIGS) vectors based on tobacco rattle virus (TRV) are now widely used for characterizing the function of plant genes. However, previous TRV vectors using RNA2 to carry the targeted gene sequence had difficulties to induce gene silencing on some plant species (*Gerbera hybrida* etc.) due to the obstacle of RNA2 movement. To achieve efficient gene silencing in those species, it is necessary to develop new TRV vectors, in which the targeted gene will be included in TRV RNA1 and the 16K gene will be replaced. Based on TRV RNA1, two new VIGS vectors M1 and M2 were developed through deletion part of 16K gene. Another mutant 16Kstop was also constructed to carry an early terminator in the 4th codon of 16K gene. The infectivity and gene silencing efficiency of the new constructs were assessed through a series of infection experiments. It was found that the infectivity of M1 and M2 was lower than wild TRV RNA1. M1 and M2 could induce PDS gene silencing on *Nicotiana benthamiana*, but their gene silencing efficiency was limited as compared with previous TRV VIGS vectors in which the PDS gene fragment was contained in RNA2. We also found that the 16K gene sequence, rather than the 16K protein, was required for efficient virus movement and accumulation.

Avainsanat — Nyckelord — Keywords

TRV, 16K gene, VIGS, *N. benthamiana*

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1 Introduction

Virus-induced gene silencing (VIGS) is a recently developed gene transcript suppression technique for functional characterization of plant genes. This approach involves cloning of a short sequence of the targeted plant gene into a viral delivery vector, which is subsequently used to infect plants. The natural defense mechanisms of the plant suppress virus replication, and also result in specific degradation of mRNAs from the targeted endogenous genes. VIGS as a reverse genetic method, its incomparable advantages on gene functions study have been reviewed previously (Burch-Smith *et al*., 2004).

Many plant RNA and DNA viruses have been modified to serve as VIGS vectors (Roberson, 2004; Burch-Smith *et al*., 2004). Among those VIGS vectors, the one based on tobacco rattle virus (TRV) is most prominent, because it overcomes the limitations of host range and meristem exclusion (Liu *et al*., 2002a; Ratcliff *et al*., 2001). TRV vectors to date have been used to silence endogenous genes in multiple Solanaceae genera including *Nicotiana* (Abbink *et al*., 2002; Jin *et al*., 2002; Liu *et al*., 2002a, b; Ratcliff *et al*., 2001), *Capsicum* (Chung *et al*., 2004; Ryu *et al*., 2004), *Solanum* (Brigneti *et al*., 2004), *Lycopersicon* (Liu *et al*, 2002b; Valentine *et al*., 2004) and *Petunia* (Chen *et al*., 2004; Ryu *et al*., 2004), as well as in *Arobidopsis* (Valentine *et al*., 2004; Wang *et al*., 2006) and *Papaver somniferum* (Hileman *et al*., 2005).

TRV, belonging to genus tobraviruse, has a bipartite, positive-stranded RNA genome. Each TRV RNA genome is encapsidated separately in a rod-shaped particle (MacFarlane, 1999). The smaller RNA (RNA2) varies considerably in size and gene content but always encodes the coat protein (CP). The larger genomic RNA (RNA1) is closely conserved in size and gene content. RNA1 encodes the 134K and 194K proteins that comprise the viral replicase. RNA1 also encodes a 29K protein involved in cell-to-cell movement of the virus (Zeigler-Graff *et al*., 1991) and a 16K cysteine-rich protein (CRP).

About the function of RNA1 16K protein, two previous reports have given entirely different results. Guilford *et al*. (1991) demonstrated that the 16K protein was not essential for TRV replication or for cell-to-cell spread. However, Liu *et al*. (2002) reported that the 16K protein was a pathogenicity determinant and was required for efficient virus

replication. They also deduced that the TRV 16K protein might be a post-transcriptional gene silencing (PTGS) suppressor.

In our previous study, using available TRV VIGS vectors (Ratcliff *et al*., 2001; Liu *et al*., 2002a), we could not get efficient gene silencing on some plant species, such as *Gerbera hybrida* and strawberry, due to the difficulty in movement of TRV RNA2, which was always designed to carry targeted gene sequence in previous TRV VIGS vectors.

In order to develop new VIGS vectors for those species, and further study the function of 16K gene, three TRV RNA1 16K gene mutants M1, M2, and 16Kstop were constructed based on TRV VIGS vector pBINTRA6 (Ratcliff *et al*., 2001). Mutant 16Kstop carries an UAG terminator in the 4^{th} codon of 16K gene to prevent the formation of a functional 16K protein. Mutant M1 is identical to the previous reported construct pTR16D4 (Guiford *et al*., 1991), in which 305 nucleotides from the 3'-part of 16K gene have been deleted. In Mutant M2, 251 nucleotides from the 5'-part of 16K gene have been deleted. Both M1 and M2 comprise a terminating codon in the beginning of 16K gene.

In this research, the infectivity of M1, M2, 16Kstop, and pBINTRA6 were assessed. M1 and M2, as new VIGS vectors, their gene silencing efficiency was also evaluated. Based on the M and NM type infection experiments using different TRV RNA1 constructs, the function of 16K gene was further studied.

2 Literature review

2.1 Plant viruses

Plant viruses were first mentioned during Tulipomania in 15th century in Holland (Matthews, 1991). However, it was until 1956, when Fraenkal-Conrat was able to isolate protein and RNA from tobacco mosaic virus (TMV) and to use it to infect healthy tobacco plants (Frankel-Conrat, 1956). Since then, more than 2,000 virus species have been identified, and nearly half of them can attack and cause disease in plants (Agrios, 2006).

True viruses usually possess the following characteristics: 1) consist of either double/single-stranded DNA or RNA molecules 2) they are protected by a coat protein which surrounds their genome 3) the virus can only survive within the infected host (Mathews, 1991). Viruses, unlike microorganisms, do not divide and do not produce any kind of specialized reproductive structures such as spores. Instead, they multiply by inducing host cells to make more virus. Viruses cause disease not by consuming cells or killing them with toxins, but by utilizing cellular substances during multiplication, taking up space in cells, and disrupting cellular processes. These in turn upset the cellular metabolism and lead to the development of abnormal substances and conditions injurious to the functions and the life of the cell or the organism.

Plant viruses can be classified into 20 groups according to following cryptograms: a) the nucleic acid type; b) the molecular weight of the nucleic acid and percentage in infective particles; c) morphology of the virus particle; and d) the type of host infected and mode of transmission (Gibbs *et al*., 1996). However those cryptograms never became widely used and have been largely abandoned. Many of the plant viruses that are recognized as distinct taxonomic entities are now grouped into genera and some of these into families. The seventh report of the International Committee for the Taxonomy of Viruses lists 17 families and 79 genera (Van Regenmortel *et al*., 2000). Virus species names are usually based on host plant and the symptom they induce.

Plant viruses appear in different sizes and shapes. Nearly half of them are elongate (rigid rods or flexuous), and many of them are spherical (isometric or polyhedral) or cylindrical bacillus-like rods (Agrios 2006). Many of plant viruses have split genomes. They may contain two or more distinct nucleic acid strands encapsidated in different-size particles. The surface of viruses consists of a definite number of protein subunits, which are arranged spirally in the enlongated viruses and packed on the side of the polyhedral particles of the spherical viruses (Agrios 2006).

The protein coat of a virus not only provides a protective sheathing for the nucleic acid of the virus, but also plays a role in determining vector transmissibility of a virus and the kinds of symptoms it causes. Protein itself has no infectivity, but serves to protect the nucleic acid and its presence generally increases the infectivity of the nucleic acid (Agrios 2006).

The infectivity of viruses is strictly the property of their genomic nucleic acid, which in most of plant viruses is RNA. Some viruses carry within them a transcriptase enzyme that they need in order to multiply and infect. However, the viral RNA contains all the genetic information needed for it to multiply and produce specific proteins.

Plant viruses enter plant cells only through wounds made mechanically or by vectors or by deposition into an ovule by an infected pollen grain. In a simplified replication of an RNA virus, the nucleic acid (RNA) of the virus is first freed from the protein coat. It then induces the cell to form the viral RNA polymerase. This enzyme utilizes the viral RNA as a template and forms complementary RNA. The first new RNA produced is not the viral RNA but a complementary copy of it. As the complementary RNA is formed, it is temporarily connected to the viral strand. Thus the two form a double-stranded RNA that soon separates to produce the original virus RNA and its complementary (-) strand, the latter then serving as a template for more virus (+strand) RNA synthesis (Scholthof et al., 1993).

The replication of some viruses differs considerably from the aforementioned scheme. In viruses in which different RNA segments are present within two or more virus particles, all the particles must be present in the same cell for the virus to replicate and for infection to develop (Agrios 2006). If the viral RNA is the (-) strand, it must be transcribed by a virus-

carried enzyme called transcriptase into a (+) strand RNA in the host, and the latter RNA then replicate as described earlier. In double-stranded RNA isometric viruses, the RNA is segmented within the same virus, is noninfectious, and depends for its replication in the host on a transcriptase enzyme also carried within the virus. On infection of a plant with a double-stranded DNA (dsDNA) virus, the viral dsDNA enters the cell nucleus, where it appears to become twisted and supercoiled and forms a minichromosome. The latter is transcribed into two single-stranded RNAs: the smaller RNA is transported to the cytoplasm, where it is translated into virus-coded proteins and the larger RNA is also transported to the same location in the cytoplasm, but it becomes encapsidated by coat protein subunits and is used as a template for reverse transcription into a complete virion dsDNA (Agrios 2006).

Viruses spread in the plant through two approaches. One is cell to cell movement through the plasmodesmata connecting adjacent cells. Viruses multiply in each parenchyma cell they infect. In leaf parenchyma cells the virus moves approximately 1 millimeter, or 8 to 10 cells, per day (Figure 1). The other spread method is systematically movement through phloem. Most viruses require 2-5 days or more to move out from an inoculated leaf (Figure 2) (Agrios 2006). Once the virus has entered the phloem, it moves rapidly in it toward growing regions (apical meristems) or other food-utilizing parts of the plant, such as tubers and rhizomes. In the phloem, the virus spreads systemically throughout the plant and reenters the parenchyma cells adjacent to the phloem through plasmodesmata. To be transmitted from plant to plant, plant viruses depend on one or more following transmission modes: 1) through vegetative propagation, 2) mechanically through the sap, 3) through seed, 4) through pollen, 5) through specific insects, mites, nematodes, or fungi.

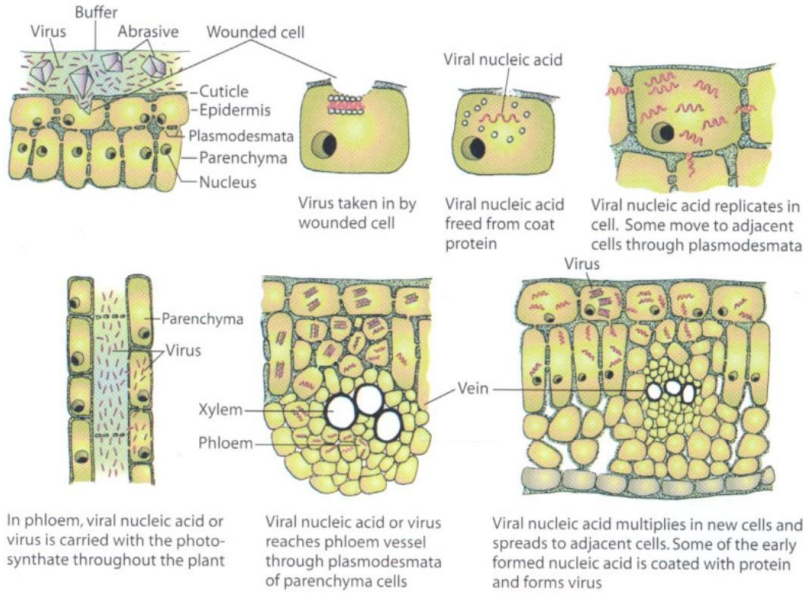

Figure 1 Mechanical inoculation and early stages in the systemic distribution of viruses in plants (Agrios, 2005, pp735).

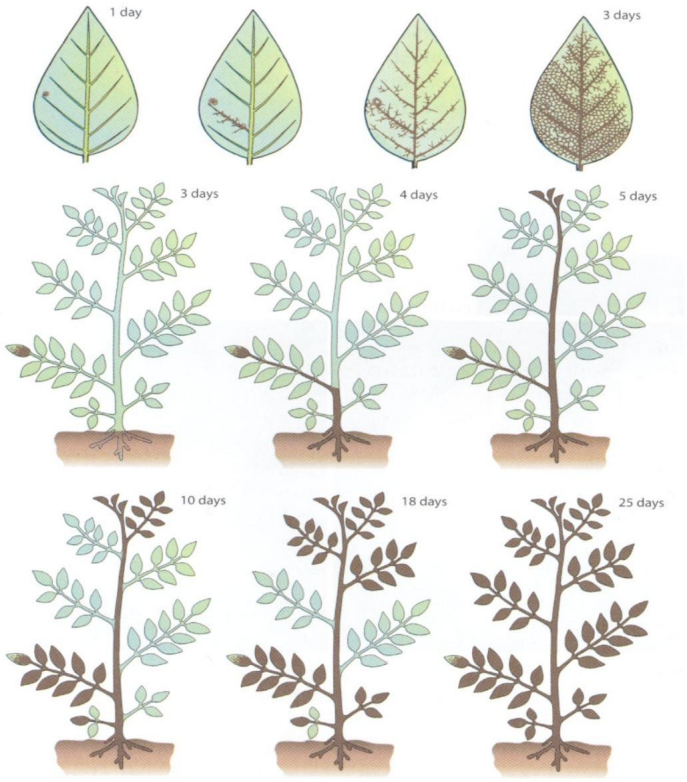

Figure 2 Schematic representation of the direction and rate of translocation of a virus in a plant. From Agrios (2006), which is modified from Samuel (1934).

2.2 Gene silencing

Gene silencing is a means to suppress gene activity at the level of mRNA expression (Cigan *et al*., 2005). It was originally discovered in petunia plants in 1990, when the transgene, encoding chalcone synthase (CHS gene) or its corresponding cDNA under the control of CaMV promoter, was introduced into petunia plant, the gene silencing phenotype came out (Napoli *et al*., 1990). Instead of deep purple blue flower, many of the transgenic flowers grew up variegated or virgin white due to gene silencing. Later gene silencing was also found to occur in transgenic tobacco plants in which a virus transgene (capsid protein of tobacco etch virus-CP of TEV) had been incorporated (Lindbo *et al*., 1993). This silencing in transgenic plants occurred because of the sequence similarity between the virus or transgene and endogenous nuclear gene.

2.2.1 Types of gene silencing

According to the time that the homologous genes suppression occurs, Mandahar (1999) divided gene silencing into two types: transcriptional gene silencing (TGS) if it occurs at the time of transcription of the transgene, and post transcriptional gene silencing (PTGS) if it occurs afterwards.

PTGS is extensively studied nowadays. It was first discovered in plants as a self-defense mechanism. It is also referred to RNA silencing in plants, quelling in fungi, and RNA interference in animals (Pantaleo *et al*., 2007).

According to the mechanisms of gene suppression, the various types of gene silencing are also divided into exogenously triggered RNA silencing, endogenous RNA silencing, and chromatin-targeted RNA silencing (Brodersen and Voinnet, 2006).

2.2.2 Mechanisms of gene silencing in plants

Gene silencing operates through different of pathways, but all processes result in sequence-specific inhibition of gene expression, either at the transcription, mRNA-stability or

translational levels. Those processes share three biochemical features (Brodersen and Voinnet, 2006): 1) formation of double-stranded (ds) RNA; 2) processing of dsRNA to small 20-26-nt dsRNAs with staggered ends; and 3) inhibitory action of a selected sRNA strand within effector complexes acting on partially or fully complementary RNA or DNA. Although several mechanisms can generate dsRNA, the sRNA processing and effector steps have a common biochemical core. sRNAs are produced by RNase III-type enzymes called Dicers. One of the two sRNA strands joins effector complexes called RNA-induced silencing complexes (RISCs), which invariably contain a member of Argonaute (ago) protein family (Brodersen and Voinnet, 2006).

At least three pathways of gene silencing are now known in plants (Dorokhov, 2007): i) exogenously triggered RNA silencing accompanied by siRNA production; ii) endogenous mRNA silencing associated with specific short double-stranded microRNA (miRNA); iii) DNA methylation and transcriptional gene silencing. All those pathways build over the Dicer-Ago core and execute biological functions including regulation of endogenous gene expression, transposon taming, viral defense and heterochromation formation.

Exogenously triggered RNA silencing pathway

There are two kinds of exogenous trigger: i) inverted-repeat (IR) transgenes; and ii) sense transcripts produced from single-copy of transgene insertions, such as viral genomic RNA. This pathway mainly happens in cytoplasm and functions mostly in transgenic and virus-infected plants (Brodersen and Voinnet, 2006).

The transcripts of IR transgenes can efficiently induce the production of dsRNAs. In the latter process, Dicer 4 cleaves dsRNAs into siRNAs (20-24 nt). At the next step, the so-called guide strand of the resulting siRNA is incorporated in RISC and, as its component, complementarily interacts with target mRNA. The other strand is removed from RISC and is degraded. The specific protein Ago1, which is the main, and possibly only protein component of the RISC, cleaves the targeted mRNA (Brodersen and Voinnet, 2006) (Figure 3).

In case of viral RNA, cells infected with viral genomic RNA produce RNA-dependent RNA polymerase (RDRP 6), which synthesizes replicative dsRNA. This RNA provides a

substrate for RNase Dicer 4. The rest processes are similar as IR-PTGS (Figure 3). It is believed until recently that the main source of siRNA is viral replicative dsRNA, which is synthesized by viral RdRP on the template of the single-stranded viral genome. Surprisingly, it was found that only 20% of siRNA is produced from dsRNA, while the other 80% originate from hairpin regions of viral genomic single-stranded RNA (Molnar *et al*., 2005; Ho *et al*., 2006)

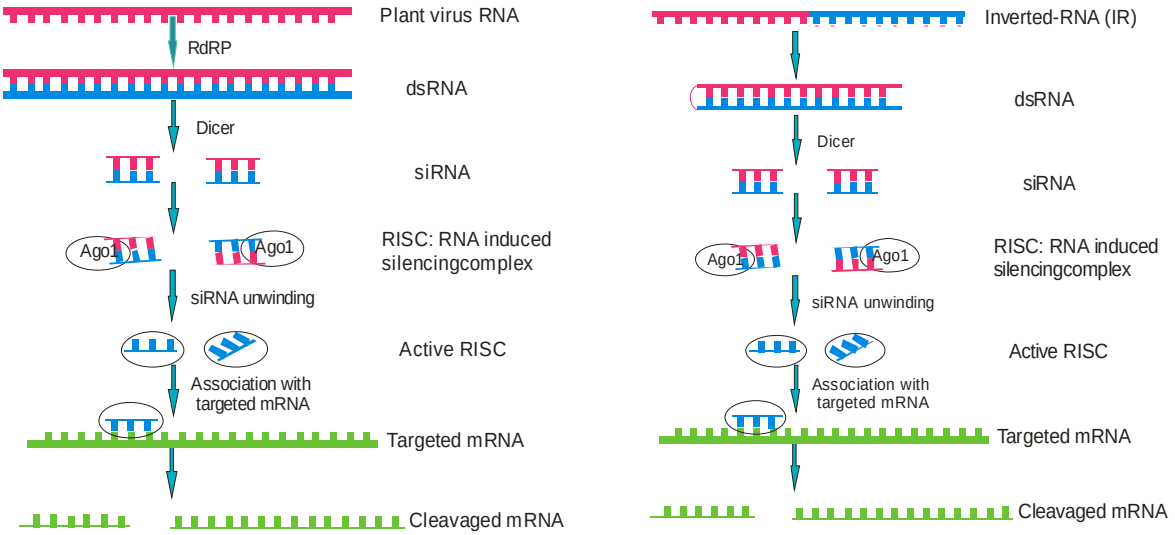

Figure 3 Exogenously triggered RNA silencing pathyway. Left: Virus induced gene silencing. With the action of RNA dependent RNA polymerase (RdRP), dsRANs are synthesized. These dsRNAs are then processed into siRNAs (20-24 nts), which will be incorporated into RNA induced silencing complex (RISC). After unwinding, RISC is associated with targeted mRNA. The special protein of RISC, Ago 1, directs mRNA degradation. Right: Inverted-Repeated (IR) transgene induced gene silencing. The IR transgenes produce ds transcripts with perfect complementary arms, which is then processed by Dicer into siRNA and direct RNA degradation. (Adapted from Brodersen and Voinnet, 2006)

Endogenous mRNA silencing pathway

Endogenous mRNA silencing is caused by miRNAs, which are processed from endogenous non-coding transcripts with an extensive fold-back structure (Jones-Rhoades *et al*., 2007). miRNAs are chemically and functionally similar to siRNAs, and are incorporated into silencing complexes that contain Ago proteins. Those silencing complexes can guide cleavage of target mRNAs (Figure 4).

miRNAs are conserved in plants. They are highly complementary to conserved target mRNAs, which allows fast and confident bioinformatics identification of plant miRNA targets (Jones-Rhoades and Bartel, 2004). Although the proteins required for siRNA and miRNA biogenesis are related and sometimes overlap, the genetic requirements for miRNA and siRNA function are partially distinct in many organisms. For example, many *Arabidopsis* siRNAs require RdRP for their biogenesis, whereas miRNAs do not (Xie *et al*., 2004). Moreover, most *Arobidopsis* miRNAs are processed by Dicer-like1, one of four Dicer-like genes in *Arabidopsis*, whereas many endogenous siRNAs require DCL 3 or DCL 4 (Gasciolli *et al*., 2005).

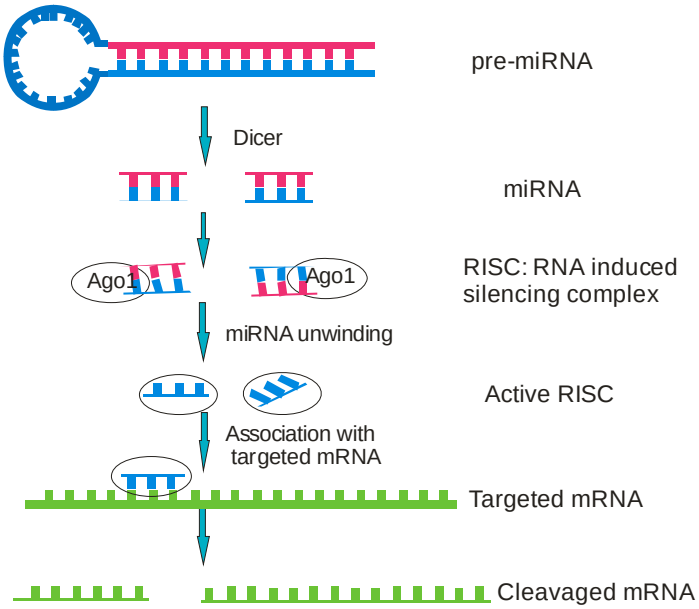

Figure 4 Endogenous RNA silencing pathway. The trigger of this type silencing is miRNAs, which originate from endogenous non-coding transcripts with an extensive fold-back structure. miRNAs are incorporated into RNA induced silencing complex, as its component, complementarily interacts with target mRNA. The special protein in RISC, Ago 1, directs the degradation of mRNA. (Adapted from Brodersen and Voinnet, 2006)

DNA methylation and transcriptional gene silencing pathway

Segments of transcriptionally silent DNA, known as heterochromatic regions, are associated with certain covalent modifications of DNA and histones. Evidence has demonstrated that siRNAs are important for establishing and/ or maintaining these heterochromatic modifications (Matzke *et al*., 2004). This transcriptional repression involves the RNA induced transcriptional silencing complex (RITS), which, like RISC, contains Ago and a single-stranded Dicer-produced siRNA, as well as Chp1 and Tas3 proteins that are not thought to be present in RISC (Motamedi *et al*., 2004).

miRNAs are also proved to be involved in the transcriptional gene silencing in plants (Bao *et al*., 2004). The interaction between miR166 (a miRNA gene) and the nascent, spliced PHB mRNA (within miR166 complementary sites) results in local DNA methylation (Bao *et al*., 2004). Although, the functional significance of this methylation change is not yet clear, methylated promoter regions are often associated with transcriptional silencing (Mette *et al*., 2005).

2.3 Virus-induced gene silencing

When viruses infect plants, the replication of their genome (either RNA or DNA) within a plant can induce it to produce siRNAs, which then directs sequence-specific degradation of virus single-stranded RNAs, and the plants will showed recovery symptoms (Waston *et al.*, 2005). It was also found that the naked genomes (without the protection of a virus particle) of a number of these viruses are infectious. The *in vitro* transcription of a cDNA clone corresponding to the complete sequence of an RNA virus can be used to generate RNA which, when rubbed onto the leaves of a plant, initiates an infection. If exogenous sequences are inserted into the virus genome, the infection of the recombinant virus could induce the silencing of inserted foreign gene, as well as the homologous endogenous genes. This phenomenon is referred as virus induced gene silencing (VIGS).

The term “Virus-induced gene silencing” (VIGS) was first used by A. van Kammen to describe the phenomenon of recovery from virus infection (van Kammen, 1997). However, the term has since been applied almost exclusively to the technique involving recombinant viruses to knock down expression of endogenous genes. Because it allows the targeted downregulation of a particular gene through the degradation of its transcripts, the potential of VIGS as a tool for the analysis of gene function was quickly recognized (Baulcombe, 1999).

2.3.1 Viruses as gene silencing vectors

Many different RNA and DNA virus have been modified to serve as vectors for gene silencing. The earliest of these vectors was based on tobacco mosaic virus (TMV), the model RNA virus (Kumagai *et al*., 1995). Transcripts of recombinant virus carrying a sequence from the *phytoene desaturase* (PDS) gene were produced *in vitro* and inoculated onto *Nicotiana benthamiana* plants to successfully silencing PDS. A more recent VIGS vector is based on potato virus X (PVX) (Ruiz *et al*., 1998). Although this vector is more stable than the TMV-based vector, PVX has a more limited host range than TMV, with only three plant families that are susceptible to PVX compared with nine families for TMV (Brunt *et al*., 1996). Furthermore, both TMV and PVX-based vectors cause disease symptoms on inoculated plants, thus making interpretation of some subtle PTGS phenotypes difficult. In addition, these viruses are excluded from the growing points or meristems of their hosts, which precludes effective silencing of genes in those tissues (Hull, 2002). The function of the virus exclusion is still unclear, although a VIGS vector based on the tobacco golden mosaic DNA virus (TGMV) has been used to successfully silence a meristem gene, *proliferating cell nuclear antigen (PCNA)* in *N. benthamiana*. However, the TGMV virus is excluded from the meristem (Peele *et al*., 2001).

With the development of VIGS vector based on tobacco rattle virus (TRV), the limitation of host ranges and meristem exclusion were overcome (Liu *et al*., 2002; Ratcliff *et al*., 2001). TRV has one of the widest host ranges among plant virus, and spread more vigorously throughout the entire plant, including meristem tissue. The overall disease-induced symptoms are often milder compared with other viruses (Gossele *et al*., 2002).

2.3.2 Mechanisms of VIGS

RNA silencing is ubiquitously triggered by dsRNA, so is virus-induced gene silencing. However, for different VIGS vectors, the sources of dsRNAs are different, and the pathways of dsRNAs generation are different (Figure 5).

Most plant viruses have genomes of positive, ssRNA that are replicated within the cytoplasm of the host. Viral RNA-dependent RNA polymerases (RDRP) synthesize

complementary negative-stranded genomic RNA, from which numerous copies of positive-stranded RNA are reproduced. Partial or complete annealing of positive and negative RNA strands constitutes the replicative from, which provides one source of dsRNA. A second source is provided by the folding of replicated, single-stranded genomic RNA, which forms secondary double-stranded structures (Figure 5). Virus with genomes of negative ssRNA follow a similar strategy, but their genomic RNA must be first copied into a complementary, plus-stranded mRNA before proteins can be synthesized (Voinnet, 2005).

Geminiviruses contain a ssDNA genome that is replicated in the nucleus through a rolling-circle mechanism that generates dsDNA intermediates, which are the templates for both replication and transcription. Transcription is bidirectional, and the presence of complementary RNA strands provides a source of dsRNA (Figure 5) (Voinnet, 2005).

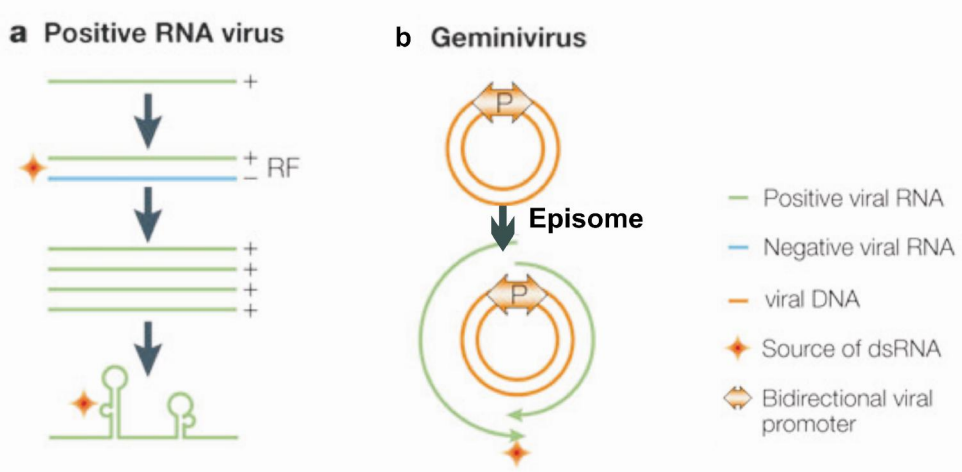

Figure 5 Different sources of dsRNA for virus-induced gene silencing. a. Viral RNA-dependent RNA polymerases synthesize complementary negative-stranded genomic RNA, from which, numerous copies of positive stranded RNA are produced. Partial or complete annealing of positive and negative RNA strands constitutes the replicative form, which provides one source of dsRNA. A second source is provided by the folding of replicated, single-stranded genomic RNA, which forms secondary double-stranded structures. b. Plant geminiviruses are ssDNA virus with genomes that are replicated in the nucleus through a rolling-circle mechanism that generates dsDNA intermediates, which are the templates for both replication and transcription. Transcription is bidirectional, and the presence of complementary RNA strands provides a cource of dsRNA. (Adapted from Voinnet, 2005)

2.3.3 Methodology of VIGS

VIGS has been widely used in reverse genetics studies by knocking down target endogenous genes. The most powerful aspect of VIGS is the minimal amount of time and effort required to identify a loss-of-function phenotype for a gene of interest (Figure

6, Burch-Smith *et al.*, 2004). Initially, single gene sequences were subcloned individually into viral genomes and plants were inoculated by rubbing leaves with viral RNA produced by *in vitro* transcription (Kumagai *et al.*, 1995). For a limited number of genes, this approach is well-suited. However, inoculation plants in this manner is time-consuming and can yield variable results. Recent efforts to streamline the cloning process and subsequent inoculation of the virus have made it possible to go from gene sequence to phenotype in plant within one month, allowing a single lab to screen thousands of individual genes for a phenotype of interest in a high-throughput manner (Lu *et al.*, 2003).

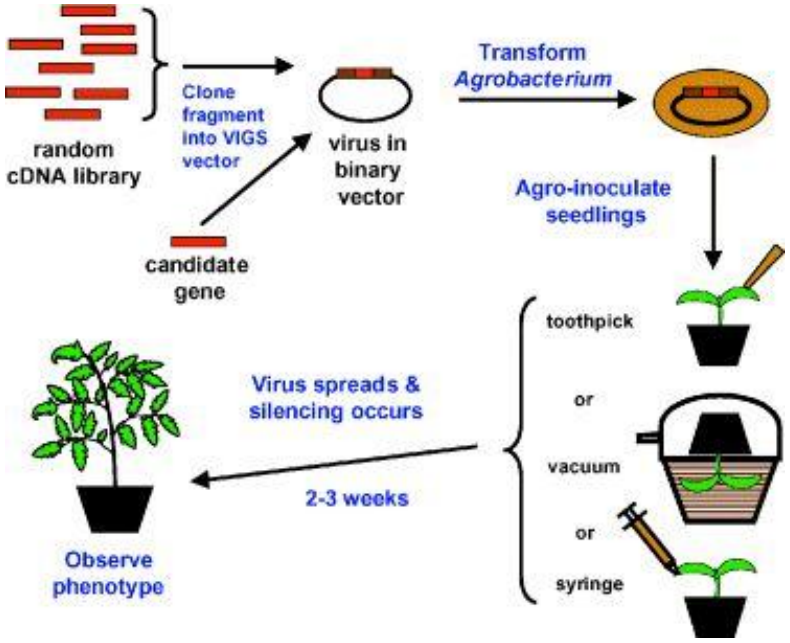

Figure 6 Method for high-throughput virus-induced gene silencing (VIGS). VIGS is performed by cloning a short stretch of sequence (usually 100–500 base pairs) from a candidate gene or random cDNAs into a virus genome under the control of the CaMV 35S promoter within a binary vector. The virus construct is transformed into *Agrobacterium* and inoculated into seedlings by a toothpick, vacuum infiltration, or syringe infiltration. The virus spreads from the site of *Agrobacterium* transformation into the upper vasculature and leaves of the growing plant, triggering a plant defense mechanism that suppresses both viral replication and expression of the endogenous host gene that was targeted. After 2–3 weeks, the plants are scored for loss-of-function phenotypes associated with suppression of the target gene. (Burch-Smith *et al.*, 2004)

Construction of vector viruses

To use a virus as a VIGS vector for target gene silencing, the first step is to subclone the given gene fragment into the viral genome. The upper limit for the size of the insert

sequence may depend on size constraints of the virus to move from cell to cell and may vary between viruses and plants. In general, the upper limit of the gene sequence is around 1.5 kb for PVX and TRV vectors used for silencing in *N. benthamiana* (Burch-Smith *et al*., 2004). Above this limit, the virus may not spread, or may lose the insert at a high rate. The lower limit of insert size has been experimentally determined to be 23 nucleotides of exact identity for transgene silencing (Thomas *et al*., 2001). Generally, fragments with length 300-500 nucleotides are adopted to ensure efficient silencing of endogenous genes.

The identity of the insert sequences to targeted endogenous gene should be carefully considered for every VIGS experiment. Because PTGS will potentially silence any transcript containing at least 23 nucleotides identity to the target sequence, it is important to determine at the outset through BLAST searches or DNA gel blot analyses, whether or not the gene to be suppressed is member of a gene family. To suppress a single gene in a family, the insert sequence should be carefully selected, so that the insert will not contain stretches of 23 nucleotides identified to other member genes. Conversely, to co-suppress several members of a gene family, it may be practical to choose regions that are conserved in those member genes (Burch-Smith *et al*., 2004).

Recent work has demonstrated that the simultaneous silencing of several distinct genes is possible by including multiple gene sequences in the virus. Peele and coworkers used a TGMV VIGS vector to co-silence a *magnesium chelatase subunit* (su) and *proliferating cell nuclear antigen* (PCNA) (Peele *et al*., 2001), whereas a VIGS vector based on Cabbage leaf curl virus (CbLCV) was used to silence *Chlorata 42* and *PDS* in Arabidopsis (Turnage *et al*., 2002). Furthermore, ligating combinations of random distinct gene sequences in the same vector could be one approach to limiting the number of plants required for large-scale screen (Burch-Smith., 2004).

Inoculation techniques

After cloning, the recombinant viruses need to be delivered into host plants for efficient infection. Several methods are employed to deliver viral silencing vectors, which include: mechanical inoculation, agroinoculation, and microprojectile bombardment (Roberson, 2005). Mechanical inoculation usually involves using in vitro transcribed RNA or extracts

from infected leaves. Mechanical inoculation is time consuming but can increase the efficiency of silencing in certain hosts such as *Arobidopsis* (Ratcliff *et al*., 2001).

Agroinoculation method has been developed for both DNA and RNA viruses, as well as transient silencing in the absence of virus. By delivering recombinant virus into plant cell with this manner, one could avoid the laborious process of producing viral transcripts *in vitro*. To date, different inoculation approaches mediated by *Agrobactirium* have been developed depending plant species and type of virus vectors. With PVX on *N. benthamiana*, sufficient infection can occur by picking the *Agrobacterium* colony with a toothpick and stabbing the toothpick into the leaf of a seedling (Lu *et al*., 2003). This is an efficient way to inoculate a large number of plants and suitable for high throughput screening. However, this method does not work well with TRV on tomato (Burch-Smith *et al*., 2004). Other agroinfiltration methods such as syringe-infiltration, vacuum-infiltration, or spraying with *Agrobacterium* were more extensively used.

Microprojectile bombardment of plasmid DNA-coated tungsten or gold micron-sized particles have been useful for inoculation of some DNA viruses (Muangsan and Robertson, 2004). However, it is more expensive compared with other inoculation approaches.

Factors influencing on the VIGS efficiency

Extensive gene silencing depends on a dynamic interplay between virus spread and plant growth, both of which can be influenced by environmental conditions. It is relatively easy to get successful gene silencing phenotypes in *N. benthamiana*. Thus, its environmental requirements are not so strict, and the normal conditions suitable for *N. benthamiana* growth could also be used as VIGS. However, for other plants, such as tomato, patato, *Arabidopsis* etc, the environmental conditions for VIGS should be more carefully controlled to get desirable silencing control.

It has been reported that under low temperature (20-22°C), low light intensity (250$\mu molm^{-2}s^{-1}$), and high humidity (85-90%), tomato plants infiltrated with TRV vectors containing PDS insert could produce extensive photobleaching phenotype (Nethra *et al*., 2005). Fu and co-workers (2006), however, reported that low temperature (15°C) and low humidity (30%) is optimal for VIGS of TRV on tomato plants. However, those environmental

conditions which are suitable for VIGS are not optimal for physiological processes of tomato. Thus, the optimization of VIGS for different VIGS vectors in different plants should be further explored.

Later, other parameters were also found to affect the efficiency of VIGS. Those parameters include: the concentration of *Agrobacterium* inocula, the inoculation techniques, the growth stage and healthy condition of inoculated plants etc (Wang *et al*., 2006).

Assessing of the silencing

The typical silencing phenotypes may appear several days to several weeks post infiltration depending on the type of virus vectors and host species, and then they become extensive throughout the infected plans (Lu *et al*., 2003). At later times they may become weak or even fade. Since the extent and lasting time of VIGS symptoms is very dependent on environment conditions, it is advisable to set up a positive control to assess the infection process. A commonly used positive control is the silencing of PDS gene, which results in photobleaching of the silenced regions and is a readily visible phenotype (Kumagai *et al*., 1995). However, it may be appropriate also to use vectors that are targeted against genes involved in the trait of interest. For example, to identity genes required for disease resistance, it would be desirable to establish progression of VIGS using a control vector that targets a known defensive-relate gene (Lu *et al*., 2003). To assess the phenotypes potentially caused by virus itself, it is also essential to include plants inoculated with empty virus as a negative control.

Some points should be realized when interpreting a VIGS phenotype (Lu *et al*., 2003). The first is that, the absence of a phenotype does not necessarily rule out involvement of the target gene in the trait of interest. VIGS is never complete and it is always possible that a silencing phenotype was not observed because the target gene function was supported by the residual low level of mRNA in the virus vector-infected plants. The second is that the observed gene silencing phenotype may result not only from the silencing of the target gene, but also others genes that have similar sequences as the insert sequence in the virus vector. Thus, the selection of the insert sequences should be very careful. It is suggested to silence the target gene again using a second nonoverlapping insert of the same gene. If the

target genes have been correctly identified, this second insert would reproduce the original VIGS phenotype (Lu *et al*., 2003).

Reverse transcription followed by realtime polymerase chain reaction (RT-PCR) is the other important method to assess the degree and specificity of VIGS. By this method, the transcript level of the targeted gene can be amplified.

Advantages and limitations of VIGS

The easiest and most effective way to determine the function of a gene or protein is to attenuate the expression of the gene or to generate a mutant that does not encode a functional protein (Burch-Smith *et al*., 2004). There are traditionally several loss-of-function approaches, such as chemical or physical mutagenesis, tilling, T-DNA insertion, and transposon activation. Nowadays VIGS has been widely used for gene function study because of its unique advantages compared to traditional functional genomic approaches (Burch-Smith *et al*., 2004).

Firstly, VIGS is rapid. It could identify a loss-of-function phenotype for a specific gene within a single plant generation. Because the gene of interest is directly targeted in VIGS, there is no need for screening large populations to identify a mutation in a specific gene. It could be finished in a month from the initial cloning to the identification of VIGS phenotype. Secondly, VIGS avoids plant transformation. VIGS is a transient method that does not rely on the generation of transgenic plants, a procedure that is difficult in many plant species. As VIGS is carried out in a young plant or mature seedling, loss of function phenotypes that would otherwise result in death at early stages of development are often avoided. Thirdly, VIGS overcomes functional redundancy. By using a targeting sequence derived from the most highly conserved region of a gene family, it is possible to silence all or most of members of a given family. Conversely, specific members of a gene family can be targeted by selecting unique sequence stretches in family members. (Burch-Smith *et al*., 2004)

There are also some limitations in VIGS as a technique for loss-of-function studies (Burch-Smith *et al*., 2004). Importantly, VIGS seldom results in the complete suppression of expression of a target gene. Therefore, because it is possible that a decreased transcript

level will still be sufficient to produce enough functional protein, a phenotype might not be observed in the silenced plant. As a result, VIGS can not rule out the involvement of a gene in a particular functional context if a phenotype is not apparent. The dependence of VIGS upon a pathogen-host interaction also presents several disadvantages. Inoculation of a plant with the virus alone can alter plant development, especially overall height and leaf morphology. As a result, it is possible that subtle phenotypes as a result of suppression of a gene could be masked by virus symptoms.

2.4 Tobacco rattle virus as VIGS vector

2.4.1 *Tobraviruses*

The genus *Tobravirus*, together with other rod-like virus genera *Tobamovirus*, *Frovirus*, *Hordeivirus*, *Pomovirus*, *Pecluvrus*, and *Benyvirus*, belongs to *Tubiviridae* family (Torrance and Mayo, 1997). *Tobravirus* includes three different viruses, tobacco rattle virus (TRV), pea early-browning virus (PEBV), and pepper ringspot virus (PepRSV) (Robinson & Harrison, 1989). TRV, in particular, has a widespread distribution, can infect a very large number of plant species and cause economically significant disease in potato, tobacco and ornamental bulbs (Brunt *et al*., 1996). PEBV has been found in northern Europe and North Africa, where it infects primarily legumes, including pea and field bean (MacFarlane, 1999). PepRSV, which has been described only in Brazil, causes disease in pepper, tomato and globe artichoke (MacFarlane, 1999).

The *tobravirus* genome includes two positive-sense, single-stranded RNAs, each of which is encapsidated separately into rod-shape particles. The larger viral RNA (RNA1) is about 6.8kb, and the smaller viral RNA (RNA2) ranges from 1.8 to 4.5 kb. The distinguishing feature of *Tobravirus* is their ability to cause two types of infection. One is NM type infection, which is caused by only the RNA1. In this type infection, RNA1 could multiply and spread in the complete absence of the second, smaller RNA2. As RNA2 encodes the coat protein (CP), infection with RNA1 alone does not produce virus particles. In the other one, M type infection, which is caused by both viral RNA1 and RNA2, the virus RNA are encapsidated (Harrison and Robinson, 1978).

Tobraviruses are unusual in being one of the only two types of virus that are transmitted from plant to plant by soil-inhabiting nematodes (Taylor & Brown, 1997). There is a highly specific relationship between virus and nematode, so that particular virus isolates are transmitted only by certain vector nematode species. The virus enters the vector as the nematode feeds on infected epidermal root cells, thus the virus is taken up with the plant cytoplasm. The distribution of *Tobravirus* depends on the distribution of the nematode vector.

2.4.2 *Tobacco rattle virus*

Tobacco rattle virus (TRV), first characterized by Quanjer (1943) (Harrison and Robinson, 1978), is an important *Tobravirus* member, which has one of the widest host ranges of any plant virus. Natural infection has been report in more than 100 species (Brunt *et al*., 1996). By inoculation with sap, about 400 species in more than 50 families can be infected (Harrison and Robinson, 1978). The disease symptoms induced by TRV include necrotic or chlorotic spots, rings, and line patterns often accompanied by variable amounts of distortion. They are considerable affected by environmental conditions.

Like other *tobravirus*, TRV also has a bipartite, positive-stranded RNA genome, and can cause M and NM type infection naturally. The infection of NM type is usually spread rapidly from cell to cell, but more slowly systemically. Correspondingly, its systemic symptoms develop slowly but more necrotic and persistent than those of M isolates (Harrison and Robinson, 1978). The viral levels of in both types of infection are initially high in TRV diseased plants, and later they decline and the infected plants enter a recovery phase. In this phase, the rate of virus replication will persist at low level and newly developing plant parts will have less severe symptoms. The plant can't be re-infected with similar virus in the recovery phase (Ratcliff *et al*., 2001).

TRV is primary soilborne through nematode spreading, but seed transmission is possible in some plant species such as tomato. TRV can persist in dormant nematodes for up to a year. Virus is spread by nematodes in a noncirculative manner and is not passed on to the nematode progeny nor does the virus stay in the vector after molting. In addition to persist

in the nematode, TRV can survive in perennial plants and in infected seeds (Vassilakos et al., 2001).

TRV has been found throughout Europe, New Zealand, in North America and Japan. Most of diseases caused by TRV are not of significant economic importance, but the TRV can cause economic losses in bulbs, such as tulip, narcissus, crocus and gladiolus (MacFarlane, 1999). Infection of potato can lower the value of the crop due to corky arcs. Vigor and yield can be decreased in tomato, tobacco, sugar beet, spinach, artichoke, celery, pepper and lettuce (Sudarshana & Berger, 1998)

2.4.3 Molecular biology of TRV

The complete infectious TRV cDNA is available for RNA1 and RNA2. According to the decreasing size of the subgenomic (sg) RNAs, two internal genes of RNA1 were denoted as 1a and 1b genes, and their translation products as the 1a and 1b proteins (MacFrlane, 1999).

RNA1 of TRV codes for four proteins of molecular masses 134-kDa, 194-kDa, 29-kDa, and 16-kDa (Figure 7 A) (Hamilton *et al*, 1987). The 134-kDa protein is analogous to the TMV 126K protein and contains domains of methyl/guanylyltransferase and helicase of superfamily 1 at the N- and C-termini, respectively. Immediately downstream of the helicase gene and in the same reading frame is a gene (194-kDa) that encodes a protein with amino acid motifs typical of RNA-dependent RNA polymerase. In vitro translation experiments suggested that this gene is expressed from full-length genomic RNA1 by readthrough translation of the terminaton codon of the helicase gene (Pelham, 1979).

The 1a gene, located downstream of the polymerase gene, encodes a 29 kDa protein that has sequence similarity to the cell-to cell-movement protein (MP) of TMV(MacFarlane, 1999). The TRV 29 kDa protein was shown to be a virus MP by mutagenesis of an infectious cDNA clone of TRV SYM RNA1 (Hamilton & Baulcombe, 1989). Deletion of the TRV 1a gene prevented accumulation of the virus in inoculated leaves of tobacco (Ziegler-Graff *et al*, 1991). However, inoculation of the TMV 1a mutant together with

TMV, or to transgenic tobacco expressing the TMV MP, did result in the accumulation of TRV in inoculated leaves (Ziegler-Graff*et al*, 1991).

The 3´-proximal gene of RNA1 encodes a 16 kDa protein. The N-terminal part of the 1b protein is rich in cysteine residues, whereas the C-terminal region includes several basic residues. Small, cysteine-rich proteins (CRPs) are encoded by a number of other plant viruses and have been implicated in virus gene expression and seed transmission. Thus far it has not been examined for any possible role in seed transmission. In earlier studies, deletion of the 16 kDa gene from TRV isolate SYM had no effect on virus accumulation in tobacco (Guiford *et al*., 1991). However, more recently, deletion of the 1b gene from TRV isolate PpK20 was shown to reduce virus accumulation greatly in both protoplasts and whole plants (Liu *et al*., 2002).

RNA2 of TRV does not have messenger activity, and the 5´-proximal CP gene is translated from a sgRNA. The corresponding sg promoter is highly conserved in TRV. The RNA1-specific genes can be located in the long 3´-terminal region of RNA2 because of identity between genomic RNAs. Additionally, In TRV strains capable of vector transmission, an ORF located downstream of the CP codes for moderately conserved 27-37 kDa protein that is required for virus transmission by root-feeding nematodes (Goulden *et al*., 1990).

2.4.4 TRV as VIGS vectors

Most of currently available VIGS vectors function only in Solanaceeous species, especially in *N. benthamiana*, whereas, barley stripe mosaic virus (BSMV) works in the monocots barley and wheat (Holzberg *et al*., 2002). Virus host-range, infectivity, and in *planta* movement are key factors on assessing VIGS vectors. VIGS vectors based on TRV possess advantages on those three aspects, and currently are widely used for gene silencing on many plants, such as *Arabidopsis*, tomato, chili pepper, and opium poppy (Wang *et al*., 2006; Liu *et al*., 2002; Chung *et al*., 2004; Hileman *et al*., 2005).

Currently, there are three versions of TRV VIGS vectors (Figure 7), which came from groups of Baulcombe (Ratcliff *et al*., 2001), Dinesh-Kumar (Liu *et al*., 2002), and Lacomme (Valentine *et al*., 2004), and they were named as TRV-B, TRV-DK, and TRV-L

respectively (Wang *et al*., 2006). Compared with TRV-B, the earliest version of TRV VIGS vector, TRV-L retains the 2b gene, while TRV-DK has a duplicated 35S promoter and ribozyme at the C-terminus, as well as some amino acid changes in the viral sequence. TRV-B-induced gene silencing in *Arobidopsis* did not give stable and satisfactory silencing when the vector was directly agroinfiltrated into *Arabidopsis* plants, and therefore a two step strategy is suggested. In this two-step procedure, the recombinant virus is first agroinfiltrated into *N. benthamiana* plants followed by sap extraction from inoculated plants and virion enrichment of the extracted sap, which is then used to inoculate *Arabidopsis* plants (Lu *et al*., 2003). This procedure is obviously time-consuming and laborious. TRV-L induces more efficient gene silencing, especially in roots of Arabidopsis when the two-step inoculation approach is employed. Compared with TRV-B, TRV-DK results in more efficient gene silencing in *N. benthamiana*, tomato, and other Solanaceous species. With the optimization of TRV-DK-induced gene silencing, Wang *et al*. (2006) have achieved consistent and highly efficient VIGS of PDS and actin in seven Arabidopsis ecotypes.

Except the broad host range, TRV vectors also have several other advantages over others VIGS vectors, such as PVX, TMV, and TGMV. First, TRV vector itself does not induce much visual symptoms that complicate a VIGS phenotype. Second, when compared with PVX, TRV-induced gene silencing affects a higher proportion of tissue and is more persistent. In addition, TRV could inhibit *Nicotiana FLO/LFY* (NFL) expression in the growing points of plants (Ratcliff *et al*., 2001), which overcomes the meristem exclusion of formal VIGS vectors.

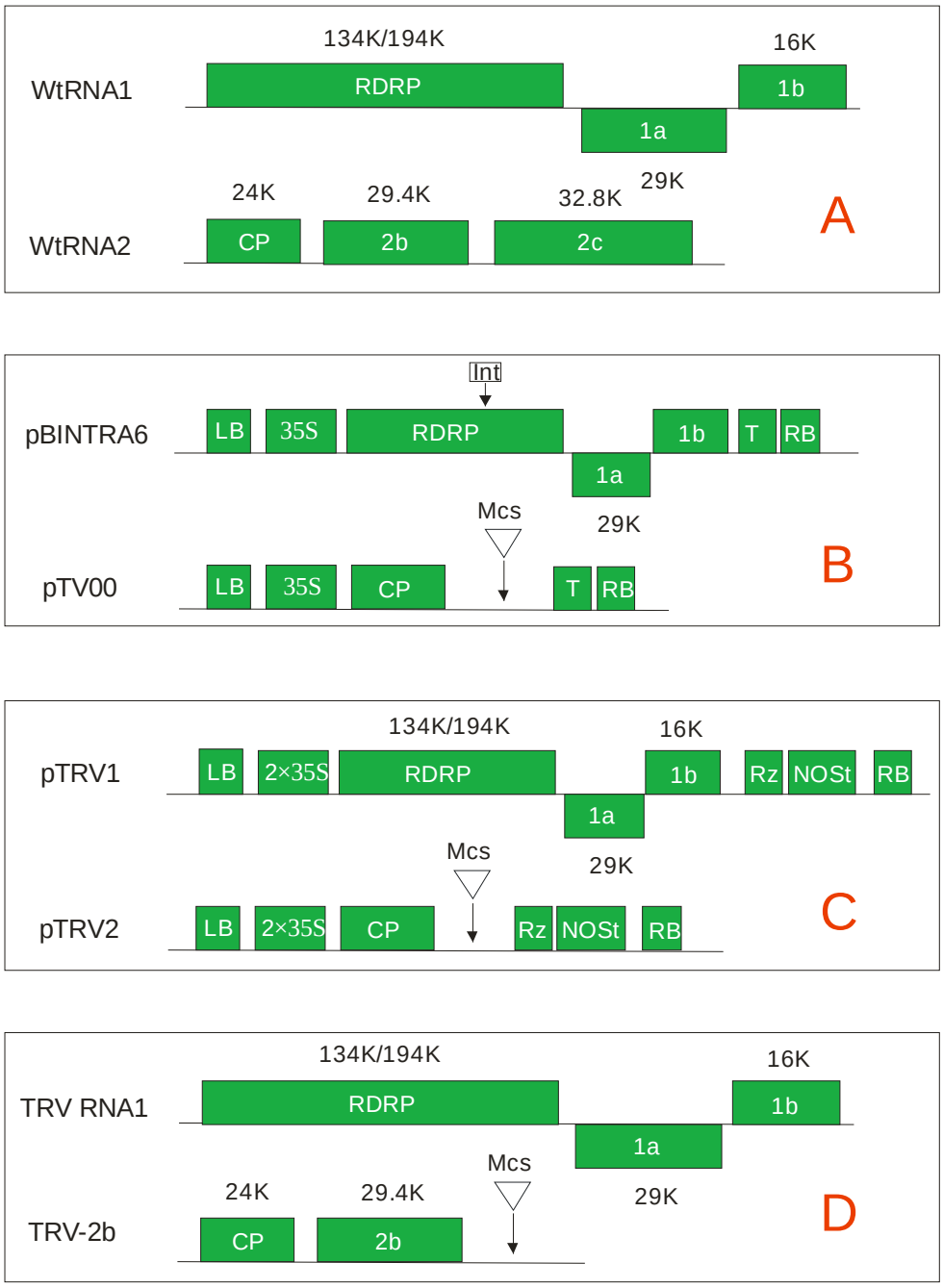

Figure 7 Genomic organization of wild TRV and TRV vectors. A) Wild TRV. RNA1 encodes the 134K and 194K polymerase, 29K movement protein, and a 16K cysteine-rich protein. RNA2 encodes a 24K coat protein, 29.4K 2b gene and a 32.8K gene (MacFarlane, 1999). B) Baulcombe's TRV vector. pBINTRA6 contained a cDNA clone of TRV RNA1, in which the RNA polymerase ORF was interrupted by intron 3 of the Arabidopsis Col-0 nitrate reductase. The cDNAclones were positioned between the left and right border (LB and RB) of the T-DNA, and between CaMV 35S promoter (35S) and transcriptional terminators (T). C) Dinesh-Kumar's TRV vector. TRV cDNA clones were placed in between the duplicated CaMV 35S promoter (2×35S) and the nopaline synthase terminator (NOSt) in a T-DNA vector. Rz refers to self-cleaving ribozyme. MCs refers to multiple cloning sites. D) Lacomme's TRV vector. In TRV-2b, the 2b gene of TRV RNA2 was kept.

3 Objectives of this research

This research is based on the previous studies of groups Baulcombe (Guiford *et al*., 1991) and MacFarlane (Liu *et al*., 2002), who have reported different result on the function of TRV 16 kDa gene. The former group thought that the protein of 16 kDa gene is not important for virus replication and accumulation. However, the later group reported that the 16-kDa protein, rather than the 16k RNA sequence is required for efficient virus replication, and thought it might be a PTGS suppressor.

The other reason to establish this research is from our previous TRV VIGS experiments on *Gerbera hybrida*. Using TRV vectors from Baulcombe group (Ratcliff *et al*., 2001) or Dinesh Kumar group (Liu *et al*., 2002), no efficient gene silencing was achieved. Sometimes, TRV RNA1 could be detected from the upper non-infiltrated leaves, but TRV RNA2 has never been detected (Timo Hytönen, unpublished results). It seems that TRV RNA2 has problems to systematically spread inside gerbera plants, which might be an important reason why efficient gene silencing was not observed on gerbera.

Thus, a new VIGS vectors based on TRV RNA1 was needed. Three TRV 16K gene mutants, 16Kstop, M1, and M2 have been constructed based on pBINTRA6. In mutant 16Kstop, the 4th codon of the 16k gene was replaced by a "TGA" terminator, so that no any protein would be produced from 16k gene sequence. Mutant M1 and M2 were further modified forms of 16Kstop. Mutant M1 is identical to pTR16D4 (GuiFord *et al*., 1991), in which 305 nucleotides in the 3'-end of 16k gene were deleted, but 12 nucleotides at 5'-end and 108 nucleotides at 3'-end were remain. Mutant M2 is the vice versa of M1, in which 251 nucleotides in the 5'-end of 16k gene were deleted, and 150 nucleotides at 5'-end and 25 nucleotides at 3'-end were remain.

The formentioned three mutants, together with pBINTRA6, were used to infect *N. benthamiana* plants in M type (with both RNA1 and RNA2 constructs) or NM type (with only RNA1 constructs). Furthermore, we used the M1:PDS and M2:PDS constructs, where the PDS gene fragments was inserted into the deletion sites of M1 and M2, to induce PDS silencing in *N. benthamiana* plants. Through those experiments, the aim was to achieve following objectives:

1. Assess the infectivity of M1 and M2 constructs.

2. Assess the gene silencing efficiency of M1 and M2 vectors.

3. Study the function of 16K gene of TRV RNA1.

4. Optimize the gene silencing conditions of TRV vectors on *N. benthamiana*.

4 Materials and methods

The general strategy of this experiment was to infect *N. benthamiana* with different TRV constructs in M (infection by both RNA1 and RNA2) or NM (infection with RNA1 constructs only) type, and analysis their infection or gene silencing status. *Agrobacterium* infiltration technique was adopted as virus inoculated method. Potato PDS gene that is homologous to *N. benthamiana* PDS gene was used as a report gene. PDS fragment (200 nucleotides) was inserted into M1, M2, and pTV00 for gene silencing. For infiltrated plants, their virus-induced disease symptoms or PDS gene silencing phenotypes were observed. The expression status of different TRV constructs was analyzed through RT-PCR and Northern blotting.

4.1 Plant material

In this research, *N. benthamiana* plants were adopted as host plants of TRV vectors. *N. benthamiana* seeds were germinated in soil in a pot (Size: 10*10cm^2) in the growth room. The pots were covered by a plastic bag to prevent drying and to provide adequate moisture. Two-week-old seedlings were transplanted individually into separate pots (Size: 8*8cm^2). Plants were infiltrated at 4-weeks old or at the 4 leaf stage.

Environment in the growth room: Day temperature: 21°C; Night temperature: 19°C; Light length: 16h per day; Light intensity: 150μmol$m^{-2}s^{-1}$. Soil was prepared by mixing peat (Kekkilä Horticulture Peat, Kekkilä Oyj) and sand with volume ratio 3:1.

4.2 Viral constructs

TRV VIGS vectors pBINTRA6 and pTV00 were kindly provided by David C. Baulcombe. TRV RNA1 mutants including 16Kstop, M1 and M2 were kindly constructed by Jani Kelloniemi (Figure 8).

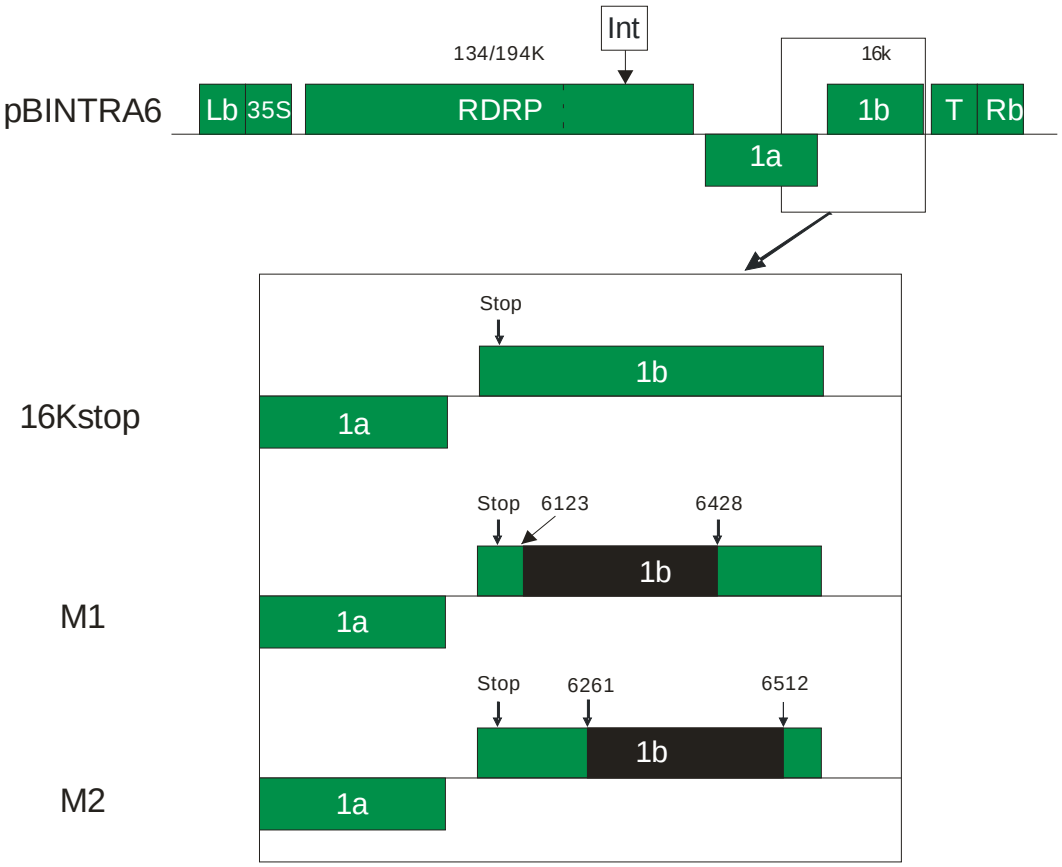

Figure 8 Genomic organization of TRV RNA1 mutants. pBINTRA6: TRV RNA1 construct containing a full length infectious cDNA clone. 16Kstop: Modified from pBINTRA6, where the 4th codon "GTA"of the 16k gene were replaced by a stop codon "TGA". M1: The modification version of 16Kstop, where 305 nucleotides of 16k gene were deleted, but 12 nucleotides at 5´-end and 108 nucleotides at 3´-end were remain. M2: The other modification version of 16Kstop, where 251 nucleotides of 16k gene were deleted, and 150 nucleotides at 5´-end and 25 nucleotides at 3´-end were remain.

4.3 PDS gene cloning

Potato PDS gene is highly homologous to *N. benthamiana* PDS gene. Thus, the available potato PDS fragment has been used as a template for PCR amplification of PDS insert. This insert, 200 nucleotides in length, was then cloned into the deletion sites of M1 and M2 to form constructs M1:PDS and M2:PDS. The flow chart of PDS gene cloning is shown in Figure 9. Construct pTV00:PDS was done by Anssi L Vuorinen through cloning a 400-nucleotide-fragment of potato PDS gene into pTV00.

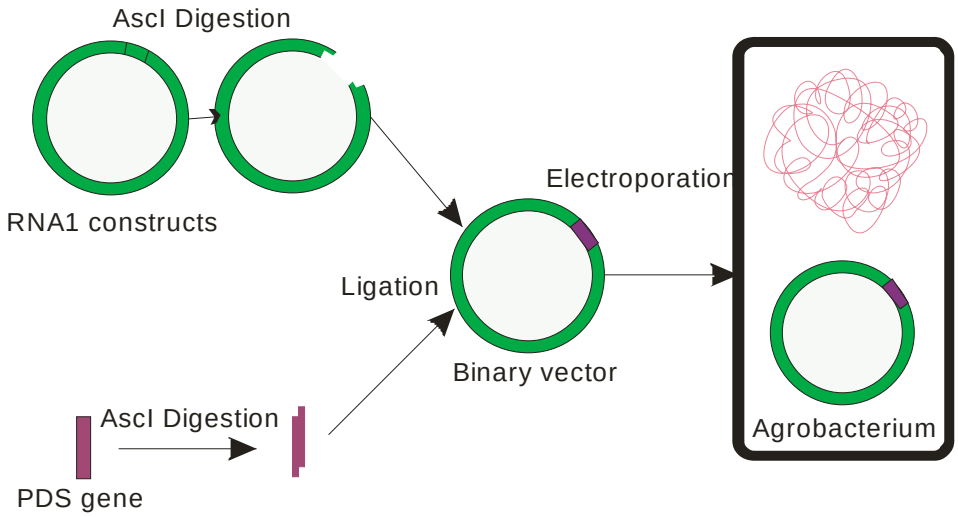

Figure 9 The flow chart of PDS gene cloning. PDS gene fragment was PCR amplified from potato PDS gene. Then both TRV RNA1 construct and PCR amplified PDS fragments were digested with Asc I enzyme. The digested PDS fragments and vector were ligated using T4 ligase. The ligated binary vector was then electroporated into *A. tumefaciens.*

Potato PDS fragment was PCR-amplified from potato (*Solanum tuberosum* cv. Pito) cDNA using Phusion polymerase (New England Biolabs, Inc.) and primers: 5'-*ATAT*ggcgcgcAACCCTGACGAACTGTCA-3' and 5'-*ATAT*ggcgcgcCCCATCCTCATTCAACTC-3'. Nucleotides in *italics* are non-PDS nucleotides added to aid the digestion enzyme to be able to work on the PCR-product. Sequence underlined is the *Asc*I digestion enzyme recognition site. The rest is potato PDS sequence. PCR was done according to the manufacturer's instruction.

The PCR fragment of PDS was check with 1% agarose gel (Appendix 2) electrophoration and purified with E.Z.N.A.^{TM} Gel Extraction Kit (Omega Bio-Tec, inc.). The purified PCR fragment and M1, M2 vectors were digested by enzyme *Asc I*. The digested PCR fragment, and M1, M2 vectors were checked with agarose gel electrophoration and purified with E.Z.N.A.^{TM} Gel Extraction Kit (Omega Bio-Tec, inc.). After digestion and purification, the digested PDS insert was ligated into M1, M2 vectors with T4 ligase. Ligation reaction (5 µl) was transformed into *E. coli* competent cells and cultured in selective LB liquid and

solid medium (Appendix 2). Positive colonies were screened by PCR and sequencing. The M1:PDS and M2:PDS binary vectors were electroporated into *A. tumefaciens* by Jani Kelloniemi.

4.4 Treatments

Three types of constructs combinations were used to achieve different aims (Table 1). Type 1 resulted in NM type infections (TRV RNA1 alone) with 4 TRV RNA1 constructs: M1, M2, 16Kstop, and pBINTRA6 (Wt RNA1). Type 2 was to obtain M type infections (with both TRV RNA1 and RNA2) including the 4 RNA1 constructs and RNA2:PDS. We expected that the infection and spread of TRV RNA1 constructs could be visible through the silencing of the reporter gene PDS. In the third type, M1:PDS and M2:PDS were used to inoculate plants for M or NM type infections. Wt RNA1 was also inoculated for M type infection as the positive control, so that the VIGS efficiency of M1 and M2 could be assessed.

Table 1: Infiltration groups and within treatments

Type 1 (NM type infection)	M1, M2, 16Kstop, Wt RNA1
Type 2 (M type infection)	M1+RNA2:PDS, M2+RNA2:PDS, 16Kstop+RNA2:PDS, WtRNA1+RNA2:PDS
Type 3	M1:PDS, M2:PDS, M1:PDS+RNA2 , M2:PDS+RNA2, WtRNA1+RNA2:PDS

4.5 Infiltration

Agroinoculation or agroinfiltration has been developed for VIGS vectors and transient silencing in the absence of virus (Kapila *et al*., 1997). To facilitate faster virus inoculation, RNA virus genomes have been placed under the control of the CaMV 35S promoter into binary vectors for *Agrobacterium*-mediated expression in plant cells. By delivering the virus into the plant cell in this manner, one could avoid the laborious process of producing

viral transcripts *in vitro*. The *agrobacterium* cells carrying the insert can be inoculated directly into the plant. Presumably there are cells at the site of inoculation that are transformed and consequently infected with the virus genome in the T-DNA. These cells would then serve as a reservoir of infection that spreads systemically throughout the plants. Different agroinfiltration methods have been developed according to the plant species and types of experiment, such as syring-infiltation, vacuum-infiltration, and spraying infiltration.

The strategies for *N. benthamiana* plants infiltration have been reported by Dinesh-Kumar et al. (2003). Most of steps on inocula preparing and plant infiltration were carried out as their description. Following are detailed steps on inocula prepare, plant infiltration, and environment control.

Preparation of inocula

1) pBINTRA6, pTV00, M1, M2, 16Kstop, M1:PDS, M2:PDS, and pTV00:PDS were separately introduced into *A. tumafeciens* strain GV2260 by electroporation. Transformants were select on LB plate containing kanamycin (50 mg/l), rifampicin (20 mg/l), and tetracyclon (4 mg/l). This step was kindly done by Jani Kelloniemi.
2) The transformants were checked the by PCR or restriction digestion, so that to confirm the presence of above TRV constructs.
3) Agrobacteria containing the above mentioned plasmids were separately inoculated into 5 ml LB media containing kanamycin (50 mg/l), rifampicin (20 mg/l), and tetracyclon (4 mg/l). The small cultures were grown at 28 ^{o}C for overnight.
4) The 5-ml overnight cultures were inoculated individually into fresh 50 ml media containing antibiotics as above. The big cultures were grown at 28 ^{o}C overnight.
5) To harvest culture bacterium, the big cultures were spun down at 3000 g for 10 min.
6) The pellets were resuspended initially in about 5 ml of infiltration media (10 mM $MgCl_2$, 10 mM MES, and 200 μM acetosyringone), and then were diluted with infiltration media to a final OD_{600} of 1.00.
7) The cultures were incubated at room temperature for 3 h.
8) In case of M type infection, *Agrobacterium* cultures containing TRV RNA1 construct and TRV RNA2 construct were mixed in 1:1 ratio.

Infiltration

1) Three *N. benthamiana* plants were infiltrated for each treatment. Two lower leaves of each plant were infiltrated using a 2-ml needless syringe.
2) The infiltrated plant was covered with newspaper over night.

Environmental conditions

To optimize environment factors for gene silencing, above treatments were replicated three times, but different growth environment was set (Table 2) for infiltrated plants.

For different round infiltration experiments, infiltrated plants were maintained under different environmental conditions (Table 2). Plants from different treatments were placed separately in different plate. Temperature, light intensity, and humidity were varied (Table 2). Plants were watered when required, and fertilizer was added into water by dissolve 1.5 ml of fertilizer (KESÄ^{TM}, Growhow Oyj) into 5 l water.

Table 2: Environmental conditions in each round infiltration

Infiltrations	Temperature (n/d)	Light intensity	Humidity
1st	19 ^{o}C/21 ^{o}C	100 μmolm^{-2}s^{-1}	30-50%
2nd	20 ^{o}C'/22 ^{o}C	150 μmolm^{-2}s^{-1}	30-50%
3rd	19 ^{o}C/21 ^{o}C	100 μmolm^{-2}s^{-1}	70-90%

4.6 Observations and sampling

Infiltrated plants were observed everyday in the morning. Every slight variation on plant growth, viral symptoms and its development, PDS gene silencing phenotypes and its development was recorded. Important viral symptoms and gene silencing phenotypes were recorded by taking photos.

Leaf samples (100-150 mg) were taken at 5, 10, 15, and 20 days post infiltration (dpi). Before sampling, photos were taken from all plants. Two top leaves, or upper non-infiltrated leaves that showed viral symptoms or gene silencing phenotypes, were sampled. Leaf samples were placed in 2-ml eppendorf tubes, frozen by liquid nitrogen immediately, and kept at -80^{o}C in freezer.

4.7 RT-PCR analysis

Viral infections were checked with reverse transcription followed by polymerase chain reaction (RT-PCR). Total RNA was extracted with TRI$_{ZOL}^{®}$ Reagent (Invitrogen^{TM}) according to the manufacturer's instructions. First strand cDNA was synthesized using 2 µg total RNA, N_8 random primer, and M-MLV reverse transcriptase. This cDNA was used as template of following PCR. Primers for TRV RNA1 detection were 5´-AGCTACTACTAGACCTAAGTC-3´ and 5´-CTATACACAGAAACAGATAAC3´, which were designed to anneal the viral sequences outside the 16k gene of TRV RNA1 (Figure 10). Primers for TRV RNA2 detection were F:5'-GGGTTACTAGCGGCACTGAA-3' and R:5'-TCGTAACCGTTGTGTTTGGA-3'. PCR products were checked by gel electrophoresis.

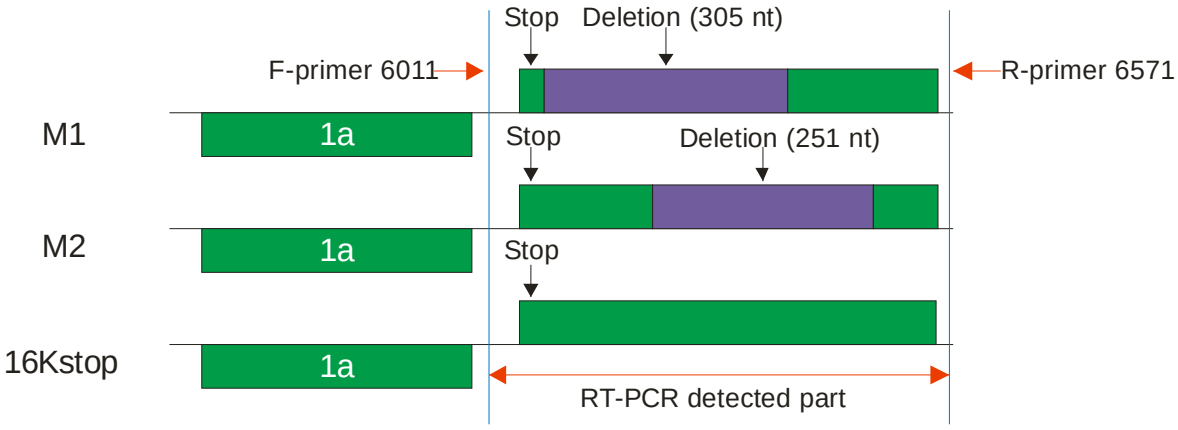

Figure 10 The detected part of M1, M2, and 16Kstop in RT-PCR. Two primers annealed to the RNA1 sequence outside of 16K gene, and sequence between 6011 and 6571 were amplified. The RT-PCR detected size of M1, M2 and 16Kstop were 255 nts, 309 nts, and 560 nts separately.

4.8 Northern-blotting

Northern-blotting was adopted to measure the amount and size of virus RNAs. Above extracted total RNA (5 μg) was fractionated in a 1.2% w/v fomaldehyde-agarose gel (Appendix 2), and transferred to Hybond-N membrane. The membrane was prehybridized, hybridized using "DIG Easy Hyb Granules" according manufacture's instruction. DNA probe was PCR amplified 46 nucleotides from 3'-UTR of RNA1, which also recognize RNA2 3'-UTR. DIG-labeled dUTP (Roche Diagnostics GmbH, Germany), *Taq* polymerase, and primers 5'-TTTGCTTTTTGATTTTATTTT-3' and 5'-CTATACACAGAAACAGATAAC-3' were used. The labeled TRV RNA1 and RNA2 were detected with "CDP-STAR, ready-to-use" (Roche Applied Science, Germany). Detailed steps are described as following:

1. RNA fractionated by gel electrophoresis

1) 1.2% FA gel of size 20×20×0.7cm3 was prepared by mix: 3.6g agrose, 30 ml 10×FA gel buffer, 260 ml RNase-free water. The mixture was heat to melt agarose, and then be cooled to 65 ^{o}C in a water bath. Subsequently, 5.4 ml of 37% (12.3 M) formaldehyde and 3 μl of a 10 mg/ml ethidium bromide stock solution was added to the mixture. After thoroughly mixing, the mixture was pour onto gel support. Before gel running, it was equilibrated in a 1×FA gel running buffer for at least 30 minutes.

2) RNA sample for FA gel electrophoresis was prepared by adding 1 volume of 5× loading buffer (Appendix 2) per 4 volumes of RNA sample. Samples were then incubated for 3-5 minutes at 65 ^{o}C, and chilled on ice, and loaded onto the equilibrated FA gel.

3) The gel was run at 5-7 V/cm in 1×FA gel running buffer for around 4 h.

2. RNA transfer from gel to membrane

1) After gel running, the gel was rinsed with DEPC-treated water, and transfered into 10 gel volumes of 20×SSC (Appendix 2) for 40 minutes.

2) After washing, the gel containing fractionated RNA was moved into a glass baking dish. Unused area of the gel was trimed away using a sharp scalpel. The gel was also cut along the slot line to allow the top of the trimmed gel to be aligned with the top of the membrane

during transfer. Then a small triangular piece was cut off from the bottom left-hand corner of the gel to simplify orientation during the succeeding operations.

3) A piece of thick blotting paper was placed on a sheet of Plexiglas or a glass to form a support that was longer and wider than the trimmed gel. It should be make sure that the ends of the blotting paper drape over the edges of the plate. The support was placed inside a large baking dish.

4) The dish was filled with the appropriate transfer buffer (20× SSC) until the level of the liquid reached almost to the top of the support. When the blotting paper on the top of the support was thoroughly wet, a glass rod or pipette was used to smooth out all air bubbles.

5) A piece of the appropriate nylon membrane (around 1 mm larger than the gel in both dimensions) was cut using a fresh scalpel or a paper cutter.

6) The nylon membrane was float on the surface of a dish of deionized water until it was wet completely from beneath, and then the membrane was immersed in 10× SSC for at least 5 minutes. A corner from the membrane was cut using a clean scalpel blade to make it match the corner cut from the gel.

7) The gel was carefully placed on the support in an inverted position so that it was centered on the wet blotting paper.

8) The gel was surrounded, but not covered, with Parafilm.

9) The top of the gel was wet with the appropriate transfer buffer. The wet nylon membrane was placed on top of the gel so that the cut corners were aligned. One edge of the membrane should extend just beyond the edge of the line of slots at the top of the gel.

10) Two pieces of thick blotting paper (cut to exactly the same size as the gel) was wet in the appropriate transfer buffer and placed then on top of the wet nylon membrane. A glass rod was used to smooth out any air bubbles.

11) A stack of paper towels (5-8 cm high) just smaller than the blotting papers was cut or fold. The towels were placed on the blotting papers. A glass plate was put on top of the stack to weight it down with a 400 g weight.

12) Upward transfer of RNA was allowed to occur for no more than 4 hours.

13) The capillary transfer system was dismantled. The positions of the slots were marked on the membrane with a ballpoint pen through the gel. The membrane was then transferred to a glass tray containing around 300 ml of 6× SSC at 23 ^{o}C. The tray was placed on a platform shaker and the membrane was agitated very slowly for 5 minutes.

14) The membrane was removed from the 6× SSC and allowed excess fluid to drain away. The membrane was then laid RNA side upward, on a dry sheet of blotting paper for a few minutes.

15) RNA was fixed by UV irradiation. The RNA side of the membrane was irradiated at 254 nm for 1 minute 45 seconds at 1.5 J/cm^2.

3. Northern hybridization

1) 40 ml of DIG Easy Hyb was preheated at 50 ^{o}C.

2) The membrane was incubated in preheated DIG Easy Hyb for 30 minutes with gentle agitation.

3) (20-50 ng/ml hybridization solution) was denatured by boiling for 10 minutes and rapidly cooling on ice-water.

4) Denatured DIG-labelled DNA probe was added to preheated DIG Easy Hyb (40 ml) and mixed well but avoid foaming (Bubbles may lead to background).

5) Prehybridization solution was poured off and immediately probe/DIG Easy Hyb mixture was added to membrane.

6) The hybridization solution was incubated with gentle agitation for at least 6 h at 50 ^{o}C

7) Hybridized membrane was washed 2× 5 minutes in ample 2× SSC, 0.1% SDS at room temperature, and 2×15 minutes in 0.5 SDS, 0.1% SDS at 68 ^{o}C under constant agitation.

4. RNA detection

1) After hybridization and stringency washes, the membrane was rinsed briefly (1-5 minutes) in washing buffer (Appendix 2).

2) The membrane was then incubated for 30 minutes in 100 ml blocking solution (Appendix 2).

3) The membrane was then incubate for 30 minutes in 20 ml antibody solution.

4) The membrane was washed 2× 15 minutes in 100 ml washing buffer.

5) The membrane was equilibrated 2-5 min in 20 ml detection buffer (Appendix 2).

6) The membrane was placed (with DNA side facing up) on a development folder and about 20 drops (0.5 ml) CDP-Star, ready-to-use solution was applied; Immediately the membrane was covered with the second sheet of the folder, and the substrate was spreaded evenly and without air bubbles over the membrane; then it was incubate for 5 minutes at 15-25 ^{o}C.

7) The membrane was exposed to a luminescent imager for 5-20 minutes.

5 Results

5.1 Phenotypes of *N. benthamiana* plants infected by TRV constructs

5.1.1 Viral symptoms caused by NM type infection

In order to assess the infectivity of the TRV RNA1 constructs, NM type infection by inoculating TRV RNA1 constructs alone was done on *N. benthamiana* plants through agro-infiltration (Table 1). Three plants were infiltrated for each treatment. And equal amount of TRV RNA1 constructs or RNA2:PDS were used.

Necrosis was the first disease symptom induced by TRV, which appeared evenly on all the infiltrated leaves of treated plants at around 3 days post infiltration (Figure 11). After that, at about 6 dpi, top leaves on 16Kstop- and Wt RNA1-infiltrated plants started curling, and then other virus related symptoms gradually appeared in the upper non-infiltrated leaves. Milder virus symptom appeared 2-3 days later on M1- and M2-infiltrated plants. On 8 dpi, viral symptom on the top leaves of WtRNA1-infiltrated plants were already very clear, whereas the top leaves of M1- and M2-infiltrated plants were still healthy-looking (Figure 11).

The observed TRV RNA1 induced disease symptoms were: a. Necrosis on the infiltrated leaves and petiole (In all treated plants); b. Necrosis on the base of the stem (In all treated plants); c. Leaf chlorosis in systemically infected leaves; d. Stunting. Sometimes the infected plants became dwarfed (serious in 16Kstop treatment plants, mild in M1 and M2 treatment plants); e. Leaf malformation, including edge curling, abnormal shape, smaller size, and uneven surface (Strong on M1, M2, and 16Kstop infiltrated plants, milder on Wt RNA infiltrated plants) (Figure 12).

From the viral symptoms showed by infected plants, it can be concluded that M1 and M2 can infect *N. benthamiana* plants and cause typical viral symptoms on them. However, viral symptoms induced by M1 and M2 appeared later and were relatively milder than those induced by WtRNA1.

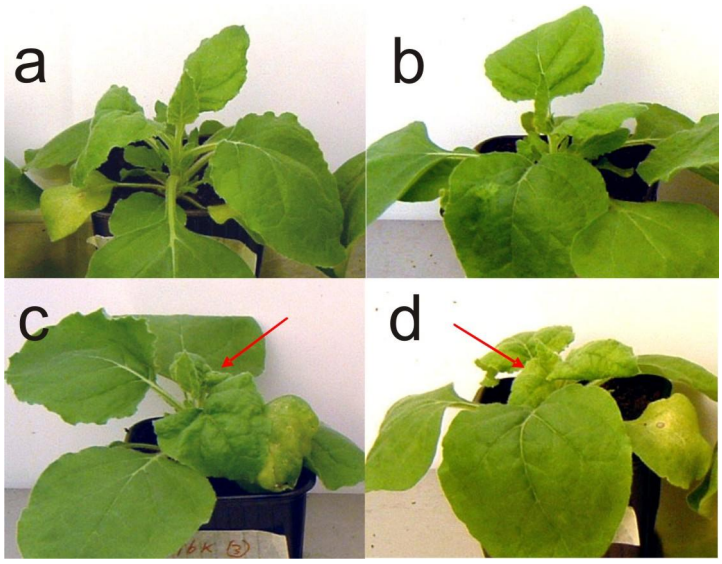

Figure 11 *N. benthamiana* plants infiltrated by TRV RNA1 constructs on 8 dpi. Necrosis was the first TRV related symptom that appeared evenly on all the infiltrated leaves of treatment plants at around 3 days. Then, at around 6 dpi, new top leaves on16Kstop- and Wt RNA1-infiltrated plants started curling. Viral symptoms on M1- and M2-infiltrated plants appeared 2-3 days later. a. M1-infiltrated plant; b. M2-infiltrated plants; c. 16Kstop-infiltrated plants; d. WtRNA1-infiltrated plants. Arrows point to top-curling-leaves.

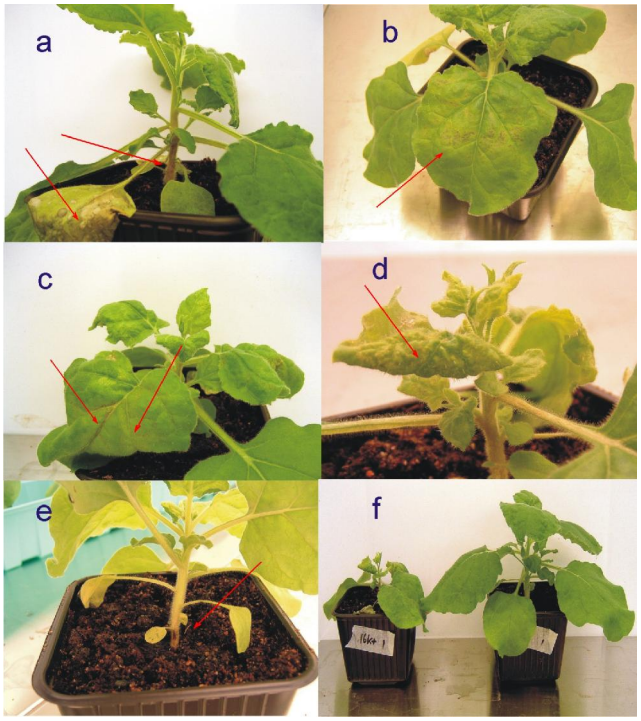

Figure 12 TRV RNA1 related virus symptoms on infected *N. benthamiana* plants on 9 dpi. a. Dark brown necrosis on petiole and base of stem (with all constructs). b. Necrosis on the leaves (in all infiltrated leaves and some upper non-infiltrated leaves). c. Yellow and dark-brown veins. d. Leaf malformation (Strong on M1, M2, and 16Kstop infiltrated plants, milder on Wt RNA1 infiltrated plants). e. Necrosis on the base of the stem. e. Growth-retarded dwarf seedling (In all infiltrated plants, but strongest on 16Kstop-infiltrated plants).

5.1.2 VIGS symptoms caused by TRV RNA1 constructs with RNA2:PDS

To further see how the modification in the 16K gene of RNA1 affect viral function, M type infection on *N. benthamiana* plants was also done through infiltrating both RNA1 constructs and RNA2:PDS (Table 1).

In this M type infection, all four TRV RNA1 constructs together with RNA2:PDS induced clear PDS gene silencing phenotypes (photobleaching) in *N. benthamiana* (Figure 13). Among 3 infiltrated plants of each treatment, 1 plant with M1+RNA2:PDS, and 1 plant with M2+RNA2:PDS showed extensive PDS gene silencing. Extensive photobleaching phenotype appeared on all plants infiltrated with WtRNA1+RNA2:PDS, and the upper leaves became totally white (Figure 13).

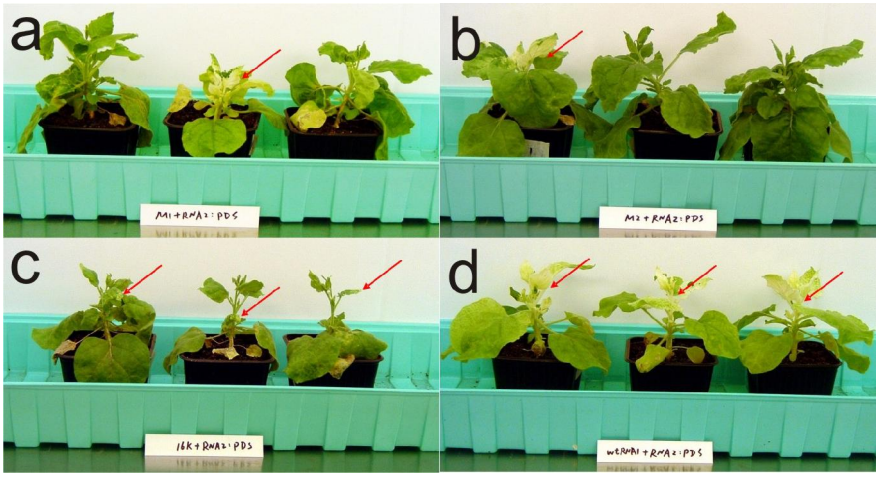

Figure 13 PDS gene silencing in *N. benthamiana* plants of 15 dpi. a. Plants infected with M1+RNA2:PDS. One of the three infiltrated plants showed extensive photo-bleaching. b. Plants infiltrated with M2+RNA2:PDS. One of three infiltrated plants showed extensive PDS gene silencing phenotype. c. Plants infiltrated with 16Kstop+RNA2:PDS. Plants in all treatment showed typical PDS gene silencing phenotypes. However, the photo-bleaching areas in upper leaves were limited. d. Plants infiltrated with WtRNA1+RNA2:PDS. All three infiltrated plants showed an extensive photo-bleaching phenotype. Arrows point to typical photo-bleaching phenotypes of PDS gene silencing.

5.1.3 Phenotypes showed by plants infiltrated with 16Kstop and 16Kstop+RNA2:PDS

Plants infiltrated by 16Kstop alone showed similar, serious viral symptoms as those infiltrated by WtRNA1 alone, and viral symptoms in the top leaves of plants appeared at

about the same time (Figure 11). Plants infiltrated by 16Kstop and RNA2:PDS showed a serious dwarf phenotype, and rather limited photobleaching compared to those infiltrated by WtRNA1 and RNA2:PDS (Figure 13).

The viral symptoms and gene silencing phenotypes of each treatment showed that TRV RNA1 constructs M1 and M2 were capable to infect *N. benthamiana* plants, but their infectivity was not so efficient as of WtRNA1 and 16Kstop. There was no significant difference in the infectivity of M1 and M2. The stop codon in 16Kstop didn't alleviate viral symptoms in infiltrated plants, however it significantly decreased the gene silencing intensity when infiltrated together with RNA2:PDS.

5.2 Molecular analysis of viral infection

To further confirm above results, viral infection in treated plants was checked using RT-PCR (reverse transcription followed by polymerase chain reaction) and northern blotting methods. Samples were taken from two top leaves at 10 dpi, whether or not they displayed symptoms, and other samples were taken from upper non-infiltrated leaves that showed viral symptoms or photobleaching phenotypes. The same amount of RNA was used for the first -stranded cDNA synthesis of RT-PCR or northern blotting.

At 10 dpi, all TRV RNA1 constructs were detected in the top leaves of treated plants (Figure 14). The accumulation levels of different TRV RNA1 constructs were different. M1 and M2 accumulated at a lower level than WtRNA1. 16Kstop accumulated in similar level as WtRNA1. The size of detected 16K gene fragments was different because of the deletion in M1 and M2. For M1, the size of RT-PCR amplified fragments was 255 nts. For M2, it was 310 nts, and for 16Kstop and WtRNA1, it was 560 nts. The size difference was visible from the agarose gel (Figure 14).

At 24 dpi, samples were taken from leaves that showed viral symptoms or PDS gene silencing phenotypes. This time, all TRV RNA1 and RNA2 constructs were also detected (Figure 15). Except for M1 and M2 in NM type infection, which accumulated in relative lower level, TRV RNA1 constructs in other treatments accumulated in similar levels. And the co-infiltrated RNA2:PDS in M type infection were also accumulated in similar levels

on all treated plants. The expected size difference of RT-PCR amplified fragments was observed (Figure 15). Northern blotting with same RNA samples gave similar results (Figure 16). However, in samples M1+RNA:PDS and M2+RNA2:PDS, extra bands corresponding to WtRNA1 were observed.

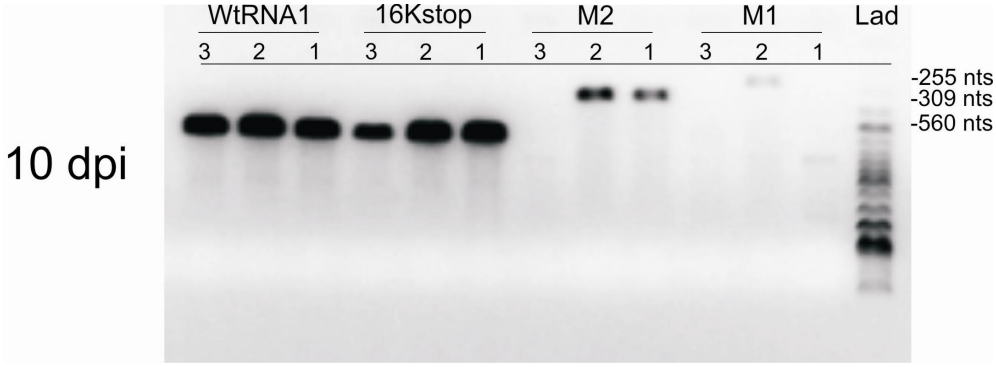

Figure 14 TRV RNA1 infection detected by RT-PCR at 10 dpi. Samples were taken from 2 top leaves. 2 µg of total RNA was used for the first strand cDNA synthesis. Three plants of each treatment were marked with 1, 2, or 3. M1, M2, 16Kstop, and WtRNA1 were the four TRV RNA1 constructs used for NM type infection. At 10 dpi, M1 and M2 accumulated in lower levels than WtRNA1. 16Kstop accumulated at similar levels as WtRNA1. The size of detected fragments was 255 nts for M1, 309 nts for M2, and 560 nts for 16Kstop and WtRNA1. Lad, molecular size marker.

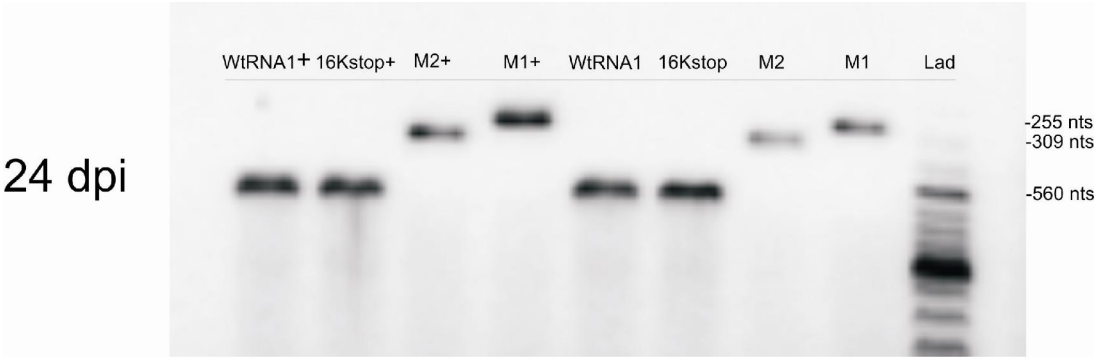

Figure 15 RT-PCR detection of TRV RNA1 at 24 dpi. Samples were taken from leaves that showed viral symptoms or photobleaching phenotypes, and 2 µg of total RNA was used for the first strand cDNA synthesis. M1, M2, 16Kstop, and WtRNA1 were the four TRV RNA1 constructs used for NM type infection. M1+, M2+, 16Kstop+, and WtRNA1+ referred to M type infection caused by M1+RNA2:PDS, M2+RNA2:PDS, 16Kstop+RNA2:PDS, and WtRNA1+RNA2:PD, respectively. The size of detected fragments was 255 nts for M1, 309 nts for M2, and 560 nts for 16Kstop and WtRNA1. Lad, molecular size marker.

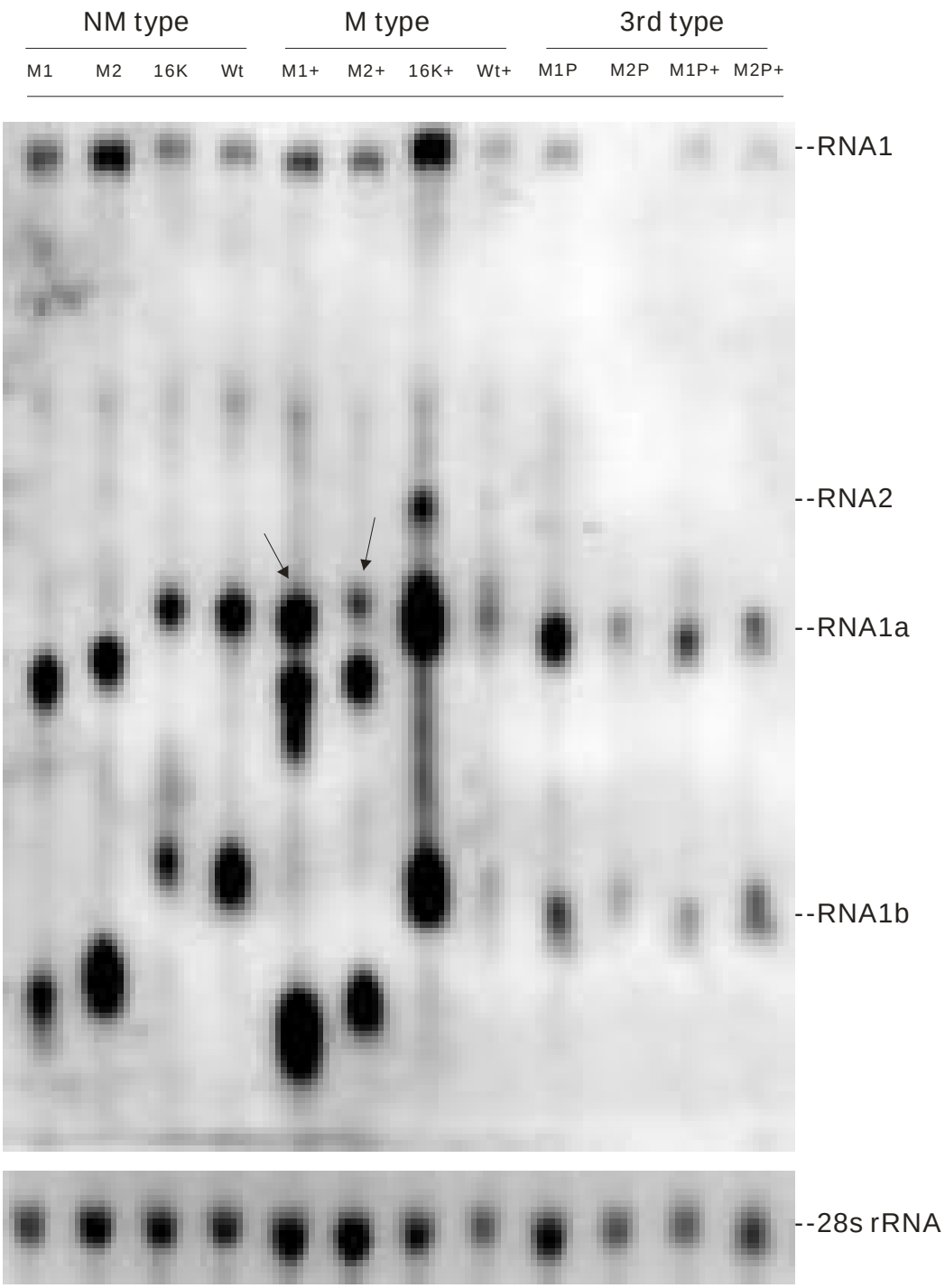

Figure 16 Northern hybridization to detect TRV constructs at 24 dpi. Samples were taken from leaves that showed viral symptoms or photobleaching phenotypes. Three RNA1s, corresponding to the genomic RNA1 (M1, 6.5 kb; M2, 6.45 kb; 16Kstop, 6.8 kb; WtRNA1, 6.8 kb), and the subgenomic RNA1-a (M1, 1.2 kb; M2, 1.15 kb; 16Kstop, 1.5 kb; WtRNA1, 1.5 kb), and RNA1-b (M1, 0.4 kb; M2, 0.35 kb; 16Kstop, 0.7 kb; WtRNA1, 0.7 kb) were detected. In NM type infection, different TRV RNA1 constructs accumulated in similar levels. In M type infection, M1, M2, 16Kstop accumulated in similar levels, whereas WtRNA1 accumulated in lower levels. When M1, M2 acted as VIGS vector to carry PDS insert, corresponding M1:PDS and M2:PDS were detected, however, they accumulated much less than M1 and M2 in the other two kinds of infection. Relative amounts of RNA in each lane are indicated by ethidium bromide staining of 28s rRNA in the smaller panels. P corresponds PDS. "+" corresponds "+RNA2:PDS". Arrows point the extra bands corresponding to contaminating WtRNA1.

5.3 VIGS efficiency of M1:PDS and M2:PDS

To test whether PDS gene silencing in *N. benthamiana* can be achieved by TRV RNA1 mutants, 200 nucleotides of PDS cDNA fragments were inserted into the deletion sites of M1, M2, as well as pTV00, to form M1:PDS, M2:PDS, and pTV00:PDS (RNA2:PDS). M1:PDS and M2:PDS were agro-infiltrated into *N. benthamiana* plants in both M and NM types of infection. As a positive control, WtRNA1 and RNA2:PDS were also agro-infiltrated into *N. benthamiana* plants of the same age.

N. benthamiana plants that were infiltrated with M1:PDS or M2:PDS, with or without RNA2 developed a mild photo-bleached phenotype on the upper non-infiltrated leaves at 15 dpi (Figure 17 A). However, *N. benthamiana* plants infiltrated with the mixture of WtRNA1 and RNA2:PDS showed extensive photo-bleached phenotype since 10 dpi (Figure 17 C). The plants infiltrated with empty *Agrobacterium* remained symptomless (Figure 17 B).

The results indicated that TRV RNA1 mutants M1 and M2 are capable of inducing gene silencing on *N. benthamiana* plants. There was no significant difference on gene silencing efficiency of M1 and M2. However, the silencing efficiency induced by M1 and M2 was not so prominent as that of WtRNA1+RNA2:PDS. In those leaves that showed photo-bleaching phenotypes, the corresponding TRV RNA1 and RNA2 constructs were detected by RT-PCR and Northern-blotting (Figure 18, Figure 16).

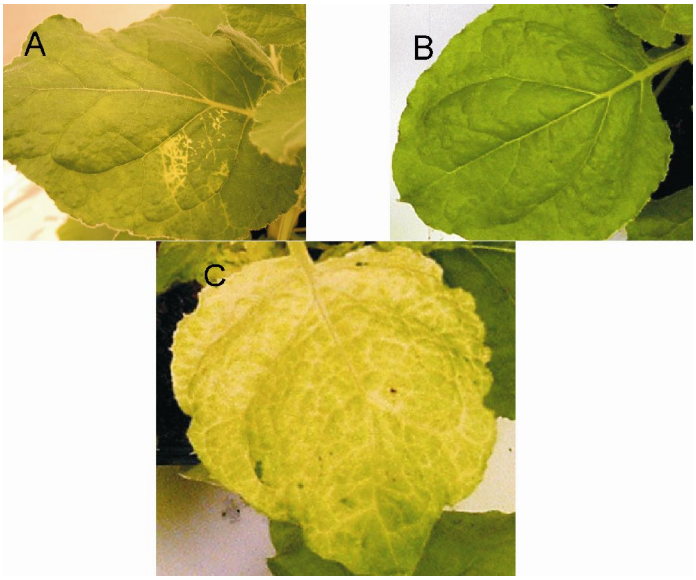

Figure 17 PDS silencing at 15 dpi. A. PDS gene silencing following systemic infection with M1:PDS. B. Healthy plant leaf. C. PDS gene silencing on plants infected by WtRNA1+RNA2:PDS.

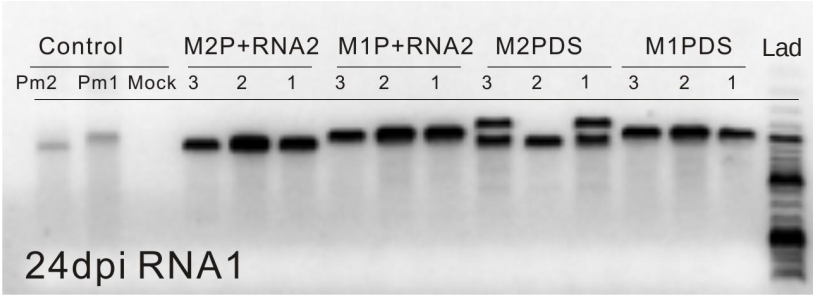

Figure 18 RT-PCR detection of M1:PDS and M2:PDS infections at 24 dpi. Samples were taken from leaves that showed photo-bleaching phenotypes at 24 dpi. Primers annealing to the 16K gene sequence were used. Three plants of each treatment were marked with 1, 2, or 3. M1P and M2P refer to M1:PDS and M2:PDS. In plants of four treatments, M1:PDS and M2:PDS were found to accumulate in similar levels. Lad, molecular size marker. Extra bands appeared in samples from M2:PDS plant 1 and 3 correspond to construct M2 without PDS insert.

5.4 Environment effects on TRV-VIGS

In order to check the environmental effects on TRV infection and gene silencing in *N. benthamiana*, three infiltration experiments were done under different environmental conditions (Table 2).

In the first experiment, the conditions were: i) temperature (day/night), 21/19°C; ii) light intensity 100 $\mu molm^{-2}s^{-1}$; iii) humidity 30-50%. The virus symptoms following M1 and M2 infections were obvious. However, the virus symptoms on M1:PDS and M2:PDS plants did not differ from mock treated plants, and PDS gene silencing phenotype (photo-bleaching) was not clear. However, plants treated with M1+RNA2:PDS and M2+RNA2:PDS showed obvious photo-bleached phenotype (Figure 19). All mutant TRV RNA1s (M1, M2, M1:PDS, M2:PDS) could be detected by RT-PCR in the upper non-infiltrated leaves (data not shown).

In the second experiment, the light intensity was increased from 100 $\mu molm^{-2}s^{-1}$ to 150 $\mu molm^{-2}s^{-1}$, and the temperature was increased from 21/19°C to 22/20°C. The humidity was kept the same as in the first experiment. The viral symptoms in this experiment were milder compared to those in the first experiment, and also the photo-bleaching phenotype in plants infected with M1+RNA2:PDS and M2+RNA2:PDS was much milder than that in the first experiment (Figure 19). All mutant TRV RNA1s (M1, M2, M1:PDS, M2:PDS) could be detected in the upper non-infiltrated leaves (Data not shown).

In the third experiment, the humidity was increased to 70-90%, but the temperature and light intensity were kept the same as in the first experiment. Virus related symptoms appeared in the infiltrated plants, and were more severe than those in the previous experiments. The photo-bleaching phenotype was also more obvious in the plants infected with M1+RNA2:PDS and M2+RNA2:PDS (Figure 19). Different from experiment 1 and 2, mild photo-bleaching phenotype appeared in the plants infected with M1:PDS and M2:PDS.

Therefore, it can be concluded that low temperature (20-22 ^{o}C), low light intensity (100 $\mu molm^{-2}s^{-1}$), and high humidity (70-90%) are favourable for TRV virus infection and virus induced gene silencing.

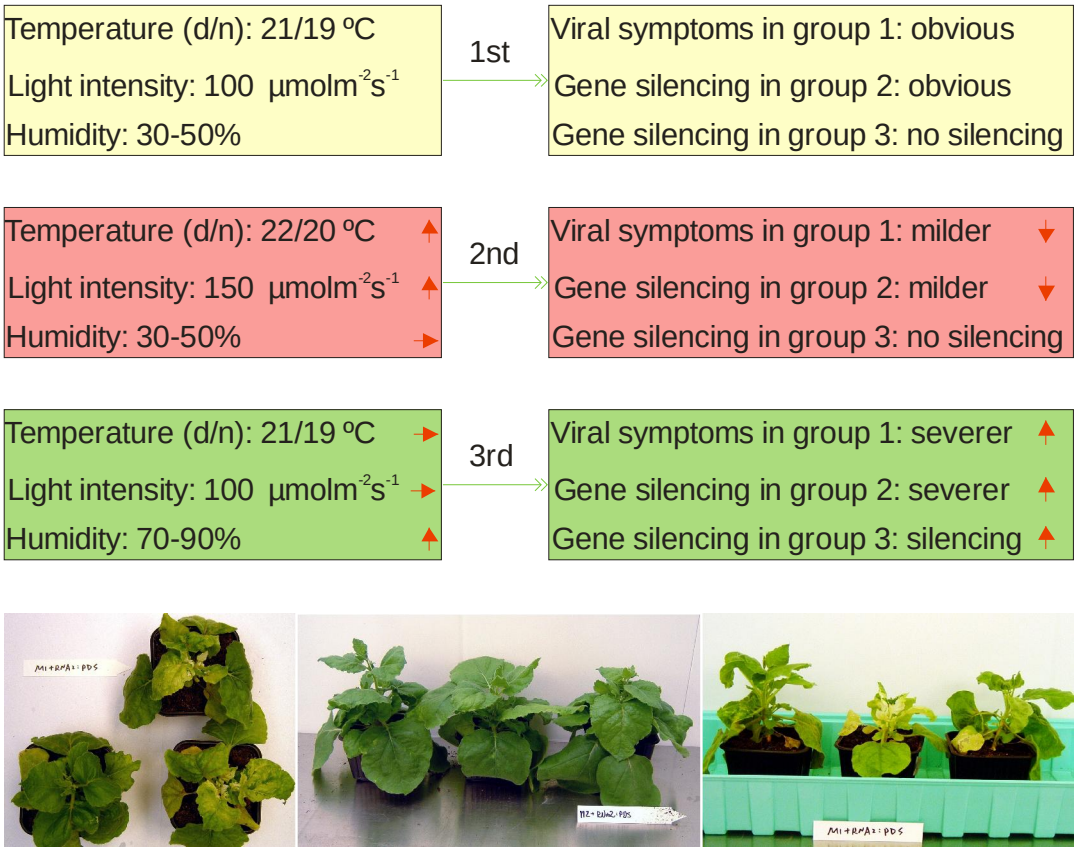

Figure 19 Environmental effects on TRV-VIGS. Low temperature (20-22 ^{o}C), low light intensity (100 µmolm^{-2}s^{-1}), and high humidity (70-90%) were favourable for TRV virus infection and virus induced gene silencing. Lower pictures show PDS gene silencing phenotypes in plants infiltrated with M1+RNA2:PDS in three experiments.

6 Discussion

6.1 16-kDa gene function

In this study, three different TRV RNA1 mutants were made and tested for their infectivity in *N. benthamiana*. In the first two mutants M1 and M2, part of 16-kDa ORF, corresponding to TRV RNA1 nucleotides 6123-6428 and 6261-6512 respectively, was deleted. In addition, the 4th codon of 16-kDa ORF was replaced with a stop codon, to avoid any truncated protein formation from the 16-kDa ORF. The third mutant 16Kstop also carried the premature translation terminator codon, but no deletion was done. Three reasons made us do the above constructs. Firstly, the aim was to use TRV RNA1 alone as a vector for gene silencing. The deletion of 16-kDa ORF could make some space for a foreign gene insert without increasing virus genome size. Secondly, as previously report, the 16k protein is a pathogenicity determinant and possibly responsible for the necrotic symptoms seen in most host plants (Liu *et al*., 2002), which make it hard to see any possible phenotypes caused by a silenced gene. Without disturbance by 16K, the virus symptoms should be milder, and the silencing phenotype was expected to be more clear. Furthermore, the 16k protein is probably the RNA-silencing suppressor of TRV (Liu *et al*., 2002), so it is expected that by disrupting 16K function the virus levels within the host plants would be lower, but still the gene silencing would be more extensive.

However, the experimental results were not fully consistent with the expectations. In NM type infection, the deletion of 16K gene in M1 and M2 slowed down their spreading and accumulation rate, while the premature translation termination codon in 16Kstop did not affect virus spreading and accumulation (Figure 14). It seems that the 16k gene sequence, but not 16k protein, is required for virus spreading and accumulation. In case of M type infection, the absence of 16 kDa protein and partial deletion of 16K gene sequence did not affect RNA2 accumulation (data not shown).

Guilford et al. (1991) also reported that the 16 kDa protein was not essential for TRV replication or for cell-to-cell spread. However, they reported that nucleotides between 6118-6428 (refers to the deletion in M1) were not essential for TRV function, which

conflicts with results of this study. The samples they used for northern blotting might have been taken from symptomatic leaves, but not from top leaves only (as we have done). As we have reported in the results part, if samples were taken from top leaves at 10 dpi, the accumulation difference of different RNA1 constructs could be detected through RT-PCR, however, if samples were taken from symptomatic leaves at 24 dpi, all the TRV RNA1 constructs were detected to accumulate in similar levels.

The Baulcombe group (Liu *et al*., 2002) reported that the 16K protein, rather than the 16K RNA sequence, was required for efficient viral replication, which is in total contrast with our results. They entirely deleted the 16K ORF (pTRV1-16Δ) and used the RNA1 and RNA2 to infect *Nicotiana tabacum* and *N. benthamiana*. At 11 dpi, pTRV1-16Δ was not detected by northern blotting and RT-PCR, which should be reasonable because even deduced from results of this study, deletion of the whole 16K gene should seriously slow down virus spreading and accumulation. They also used pTRV-16stop (very similar to 16Kstop of this study) to infected *Nicotiana benthamiana*, but they only detected very limited pTRV-16stop accumulation (they did not mention when and where they took samples), which conflicted with our results.

The gene silencing efficiency of M1 and M2 was not as expected. Although all forms TRV RNA1s and RNA2s could be detected in the upper non-infiltrated leaves, and there was no significant difference on their accumulation levels (Figure 15, Figure 16), the silencing phenotype in plants infiltrated with M1:PDS (with or without RNA2), M2:PDS (with or without RNA2), M1+RNA2:PDS, M2+RNA2:PDS, and 16Kstop+RNA2:PDS was much milder than that following infiltration with Wt+RNA2:PDS. It is possible that the 16-kDa protein is required for efficient gene silencing, which is also opposite to the previous hypothesis that 16-kDa gene might be a gene silencing suppressor (Liu *et al*., 2002).

6.2 Gene silencing efficiency of M1:PDS and M2:PDS

The results showed that TRV RNA1 constructs M1 and M2 could induce PDS gene silencing in *N. benthamiana* plants. However, gene silencing efficiency of M1 and M2 was not satisfactory as compared with Baulcombe's TRV vector (WtRNA1+RNA2). Why M1

and M2 could not induce as efficient gene silencing as Baulcombe's vector? There are several possible reasons.

Firstly, part of the 16k gene in M1 and M2 was deleted. As shown by results, the sequence of the 16K gene is required for efficient virus spreading and accumulation. The partial deletion of 16K gene sequence affected viral functions. *N. benthamiana* plants infected with M1 or M2 showed only mild virus symptoms in contrast to those infected with WtRNA1. M1 and M2 accumulation levels were also lower than WtRNA1 in the top leaves of infiltrated plants.

Secondly, because of the premature translation termination codons in M1, and M2, no protein was translated from the16K ORF. However, as already mentioned, 16K protein may be required for efficient gene silencing.

Thirdly, the insert of foreign gene sequence (200 PDS nts) affected virus replication and spreading. Unlike M1 and M2 which could be often detected in the top leaves of infiltrated plants, M1:PDS and RNA2:PDS were seldom detected by RT-PCR in the top leaves of infiltrated plants (data not shown).

6.3 Virus symptoms and VIGS phenotype

Virus symptoms, to a certain degree, reflect the situation of virus infection and accumulation in host plants. On the other hand, VIGS phenotype reflects the degree of silencing of the virus and the host gene it contains. The data showed that when photobleaching phenotype in the host plant was extensive, the virus symptoms were always mild. It seems that the virus-induced gene silencing decreased virus accumulation in the host plants. Northern hybridization results further confirmed this. Plants infected with WtRNA1+RNA2:PDS showed the most extensive photo-bleaching phenotype (Figure 17). However, virus in those plants accumulated in the lowest level among those of M type infections (Figure 16).

M1:PDS and M2:PDS could induce mild photobleaching phenotype in infiltrated *N. benthamiana* plants. Not like plants infiltrated with Wt+RNA2:PDS, which showed rather

extensive photobleaching in almost all plants and leaves, photobleaching in plants infiltrated with M1:PDS and M2:PDS (with or without RNA2) only appeared on 1 or 2 upper non-infiltrated leaves of the whole plants, and the silencing area in the leave was rather limited. Similar accumulation levels of corresponding TRV RNA1s (Wt RNA1, M1:PDS and M2:PDS) could be detected in the upper non-infiltrated leaves (Figure 16).

Regretfully, the silenced endogenous PDS gene levels, which should directly show the gene silencing level, were not measured. In the future experiments, to measure the gene silencing efficiency of M1 and M2, except for observing the silencing phenotypes and virus infection, the targeted gene expression level should also be measured, and other functional gene should be tested.

6.4 Factors affecting VIGS

It has been reported that the environmental conditions greatly affect gene silencing efficiency, and the optimum environment for gene silencing could vary from species to species (Szittya *et al*, 2003; Nethra *et al*., 2006; Fu *et al*., 2006; Wang *et al*., 2006). Usually, the favourable environmental conditions for the plant growth are not optimal for VIGS. For *N. benthamiana*, it is reported that low temperature (less than 24 ^{o}C), low light intensity (less than 250 $\mu molm^{-2}s^{-1}$) and high humidity (85-90%) could enhance VIGS (Nethra et al., 2006). In this study, three experiments were done with different light intensity and humidity. When light intensity was increased from 100 $\mu molm^{-2}s^{-1}$ to 150 $\mu molm^{-2}s^{-1}$, PDS silencing efficiency decreased. On the other hand, when humidity was increased from 30-50% to 70-90% and light intensity was kept as 100 $\mu molm^{-2}s^{-1}$, PDS VIGS efficiency significantly increased, which is consistent with the previous report by Nethra (2006).

Other factors, such as the inoculation method, the growth stage of the experimental plants, the concentration of *Agrobacterium* inocula, and the space for the roots development (the size of growth pot), were also reported to affect the VIGS efficiency (Robertson, 2004; Wang *et al*., 2006). To use M1 and M2 for VIGS would require further study for determination of the optimal factors to efficient VIGS.

6.5 Sampling

It was noticed that sampling is a very important factor because the virus does not uniformly spread in the whole plant, and its accumulation levels are not constant. One should be very careful to decide when and where to take leaf samples to assess the virus spreading and accumulation situation,

The different TRV RNA1 constructs spread systematically with different speed. For WtRNA1 and 16Kstop, it took 5-6 days to spread from infiltrated leaves to top leaves, whereas for M1 and M2, it took 7-8 days to spread from infiltrated leaves to top leaves. At around 15 dpi, viral accumulation in the top leaves started to decrease, and some leaves recovered (virus could be no longer detected by RT-PCR).

Samples should be taken at different times and from different parts of the plants. In this study, to assess virus movement and accumulation in *N. benthamiana*, taking samples from two top leaves at 5 dpi and 10 dpi was advisable. However, if one would like to assess the viral replication, leaf samples should be taken from every part of plant at 5 dpi, 10 dpi, and 15 dpi.

6.6 Contamination problems

VIGS experiment included many steps, such as cloning of the mutant TRV RNA1, cloning of gene fragment, binary *Agrobacterium* vector construction, infiltration, result assessment etc. To get precise results, every step should be done very carefully. Throughout the experiment, the most important thing was to keep all *Agrobacterium* cultures (containing different TRV RNA1 and RNA2 forms) isolated. It is suggested to check the purity degree of the *Agrobacterium* cultures when they have been transformed. During the *Agrobacterium* cultivation, harvest, mix, and infiltration, any contamination should be prevented. Even after infiltration, when the infiltrated plants are already in the growth room, it is possible that different *Agrobacterium* cultures could be mixed by touching, watering etc.

In this study, some unexpected bands appeared in the RT-PCR and northern hybridization experiments. One is in Figure 18, two samples from plants infiltrated with M2:PDS contained extra shorter bands corresponding to empty M2. This might be because M2:PDS has lost its PDS insert during multiplication, which should not affect virus symptoms and VIGS. The other case was in the northern hybridization results (Figure 16) where bands corresponding to Wt RNA1 were detected in two samples from plants infiltrated with M1+RNA1:PDS and M2+RNA2:PDS separately. The contamination with WtRNA1 affected the phenotype of these two plants. They showed extensive photobleaching phenotype similar to Wt+RNA2:PDS infiltrated plants.

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Xianbao Deng

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Appendix 1. TRV isolate PpK20 sequence

RNA1 (NC_003805)

```
   1 ataaaacatt tcaatccttt gaacgcggta gaacgtgcta attggatttt ggtgagaacg
  61 cggtagaacg tacttatcac ctacagtttt attttgtttt tctttttggt ttaatctatc
 121 cagcttagta ccgagtgggg gaaagtgact ggtgtgccta aaacctttc tttgatactt
 181 tgtaaaaata catacagata caatggcgaa cggtaacttc aagttgtctc aattgctcaa
 241 tgtggacgag atgtctgctg agcagaggag tcatttcttt gacttgatgc tgactaaacc
 301 tgattgtgag atcgggcaaa tgatgcaaag agttgttgtt gataaagtcg atgacatgat
 361 tagagaaaga aagactaaag atccagtgat tgttcatgaa gttctttctc agaaggaaca
 421 gaacaagtta atggaaattt atcctgaatt caatatcgtg tttaaagacg acaaaaacat
 481 ggttcatggg tttgcggctg ctgagcgaaa actacaagct ttattgcttt tagatagagt
 541 tcctgctctg caagaggtgg atgacatcgg tggtcaatgg tcgttttggg taactagagg
 601 tgagaaaagg attcattcct gttgtccaaa tctagatatt cgggatgatc agagagaaat
 661 ttctcgacag atatttctta ctgctattgg tgatcaagct agaagtggta agagacagat
 721 gtcggagaat gagctgtgga tgtatgacca atttcgtaaa aatattgctg cgcctaacgc
 781 ggttaggtgc aataatacat atcacggttg tacatgtagg ggtttttctg atggtaagaa
 841 gaaaggcgcg cagtatgcga tagctcttca cagcctgtat gacttcaagt tgaaagactt
 901 gatggctact atggttgaga agaaaactaa agtgggtcat gctgctatgc tttttgctcc
 961 tgaaagtatg ttagtggacg aaggtccatt accttctgtt gacggttact acatgaagaa
1021 gaacgggaag atctatttcg gttttgagaa agatccttcc ttttcttaca ttcatgactg
1081 ggaagagtac aagaagtatc tactggggaa gccagtgagt taccaaggga atgtgttcta
1141 cttcgaaccg tggcaggtga gaggagacac gatgcttttt tcgatctaca ggatagctgg
1201 agttccgagg aggtctctat catcgcaaga gtactaccga agaatatata tcagtagatg
1261 ggaaaacatg gttgttgtcc caattttcga tctggtcgaa tcaacgcgag agttggtcaa
1321 gaaagacctg tttgtagaga aacaattcat ggacaagtgt ttggattaca tagctaggtt
1381 atctgaccag cagctgacca taagcaatgt taaatcatat ttgagttcaa ataattgggt
1441 cttattcata aacggggcgg ccgtgaagaa caagcaaagt gtagattctc gagatttaca
1501 gttgttggct caaactttgc tagtgaagga acaagtggcg agacctgtca tgagggagtt
1561 gcgtgaagca attctgactg agacgaaacc tatcacgtca ttgactgatg tgctgggttt
1621 aatatcaaga aaaatgtgga agcagtttgc taacaagatc gcagtcggcg gattcgttgg
1681 catggttggt actctaattg gattctatcc aaagaaggta ctaacctggg cgaaggacac
1741 accaaatggt ccagaactat gttacgagaa ctcgcacaaa accaaggtga tagtatttct
1801 gagtgttgtg tatgccattg gaggaatcac gcttatgcgt cgagacatcc gagatggact
1861 ggtgaaaaaa ctatgtgata tgtttgatat caaacggggg gcccatgtct tagacgttga
1921 gaatccgtgc cgctattatg atatcaacga tttctttagc agtctgtatt cggcatctga
1981 gtccggtgag accgttttac cagatttatc cgaggtaaaa gccaagtctg ataagttatt
2041 gcagcagaag aaagaaatcg ctgacgagtt tctaagtgca aaattctcta actattctgg
2101 cagttcggtg agaacttctc caccatcggt ggtcggttca tctcgaagcg gactgggtct
2161 gttgttggaa gacagtaacg tgctgaccca agctagagtt ggagtttcaa gaaaggtagc
2221 cgatgaggag atcatggagc agtttctgag tggtcttatt gacactgaag cagaaattga
2281 cgaggttgtt ccagcctttt cagctgaatg tgaaagaggg gaaacaagcg gtacaaaggt
```

2341 gttgtgtaac cttttaacgc caccaggatt tgagaacgtg ttgccagctg tcaaaccttt
2401 ggtcagcaaa ggaaaaacgg tcaaacgtgt cgattacttc caagtgatgg gaggtgagag
2461 attaccaaaa aggccggttg tcagtggaga cgattctgtg gacgctagaa gagagtttct
2521 gtactactta gatgcggaga gagtcgctca aaatgatgaa attatgtctc tgtatcgtga
2581 ctattcgaga ggagttattc gaactggagg tcagaattac ccgcacggac tgggagtgtg
2641 ggatgtggag atgaagaact ggtgcatacg tccagtggtc actgaacatg cttatgtgtc
2701 caacccagac aaacgtatgg atgattggtc gggatactta gaagtggctg tttgggaacg
2761 aggtatgttg gtcaacgact tcgcggtcga aaggatgagt gattatgtca tagtttgcga
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Appendix 2. Media and buffers

Liquid LB medium

Bacto-Tryptone	10g
Bacto-yeast extract	5 g
NaCl	10 g

Add ddH2O to 1 litre

Total volume 1 litre

Note: adjust pH to 7.0 and autoclave to sterilize

Plate LB medium

Bacto-Tryptone	10 g
Bacto-yeast extract	5 g
NaCl	10 g
Bacto-agar	15 g

Add ddH2O to 1 liter

Total volume 1 litre

Note: adjust pH to 7.0 and autoclave to sterilize

1% agarose gel (100 ml)

Powdered agarose	1 g
1X TAE buffer	100 ml

Heat the solution in microwave oven until agarose dissolves totally, when solution is cooled to around 65 ℃, add ethidium bromide to a final concentration of 0.5 μg/ml and mix.

8X TAE buffer (1 liter)

Tris base	38.4 g
Glacial acetic acid	9.1 ml
0.5 M EDTA (pH 8.0)	16 ml

6X DNA loading buffer

0.25% bromophenol blue

0.25% xylene cyanol FF

15% ficoll in water

10X FA gel buffer

200 mM 3-[N-morpholino]propanesulfonic acid (MOPS) (free acid)

50 mM sodium acetate

10 mM EDTA

pH to 7.0 with NaOH

1X FA gel buffer

100 ml 10X FA gel buffer

20 ml 37% (12.3 M) formaldehyde

880 ml RNase-free water

5X RNA loading buffer

16 µl saturated aqueous bromophenol blue solution

80 µl 500 mM EDTA, pH 8.0

720 µl 37% (12.3 M) formaldehyde

2 ml 100% glycerol

3084 µl formamide

4 ml 10X FA gel buffer

RNase-free water to 10 ml

20X SSC

Sodium Chloride 3M

Sodium Citrate 0.3M

pH to 7.0

Washing buffer for Northern-blotting

0.1 M maleic acid

0.15 M NaCl

0.3% Tween 20

pH 7.5 (20 °C)

Maleic acid buffer

0.1M maleic acid

0.15M NaCl

Adjust with NaOH to pH 7.5 (20 °C)

Blocking solution for Northern-blotting

10% Blocking reagent (w/v) in maleic acid buffer

Detection buffer for Northern-blotting

0.1 M Tris-HCl

0.1 M NaCl

pH 9.5 (20 °C)