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Department of Agricultural Sciences
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University of Helsinki

Finnish Doctoral Program in Plant Science

Development and Application of *Tobacco Rattle Virus* Induced Gene Silencing in *Gerbera hybrida*

Xianbao Deng

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Agriculture and Forestry, University of Helsinki, for public examination in lecture room B2, B-building (Latokartanonkaari 7-9), on 22 November 2013, at 12 o'clock noon.

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Cover figure: Viruse-induced gene silencing (VIGS) phenotypes in *Gerbera hybrida*. Left image: Silencing of the gerbera *phytoene desaturase* (*GPDS*) gene in the gerbera cultivar Terraregina. Middle and right images: Silencing of the gerbera *chalcone synthase 1* (*GCHS1*) gene in the gerbera cultivar President at the petal developmental stage 5 (middle) and stage 8 (right).

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List of original publications

This thesis is based on the following publications, which are referred to in the text by their roman numerals. The publications have been reprinted with the kind permission of the publishers.

I **Deng X**, Kelloniemi J, Haikonen T, Vuorinen AL, Elomaa P, Teeri TH, Valkonen JPT. 2013. Modification of *Tobacco rattle virus* RNA1 to serve as a VIGS vector reveals that the 29K movement protein is an RNA silencing suppressor of the virus. *Molecular Plant-Microbe Interactions* **26**: 503–514.

II **Deng X**, Elomaa P, Nguyen CX, Hytönen T, Valkonen JPT, Teeri TH. 2012. Virus-induced gene silencing for Asteraceae─a reverse genetics approach for functional genomics in *Gerbera hybrida*. *Plant Biotechnology Journal* **10**: 970–978.

III **Deng X,** Bashandy H, Ainasoja M, Kontturi J, Pietiäinen M, Laitinen RAE, Albert VA, Valkonen JPT, Elomaa P, Teeri T. Functional diversification of duplicated chalcone synthase genes in anthocyanin biosynthesis of *Gerbera hybrida*. *New Phytologist*, in press.

My contribution to the above publications:

I I was involved in the experimental design, constructed the trv30K16Kstop mutants, and carried out all the experiments. I was involved in the data interpretation, wrote the manuscript draft and revised the manuscript together with other co-authors.

II I was involved in the experimental design, screened gerbera cultivars that are sensitive to TRV infection and carried out all VIGS experiments. I wrote the manuscript draft and revised the manuscript together with other co-authors.

III I was involved in the experimental design, analyzed gerbera *CHS* sequences and carried out the experiments on *CHS* expression analysis, *in situ* hybridization, VIGS, Western blotting, and polysome RNA analysis. I wrote the manuscript draft and revised the manuscript together with other co-authors.

Abbreviations

AGO	ARGONAUTE protein
CHL	Mg-chelatase
CHS	Chalcone synthase
CP	Coat protein
DCL	Dicer-like protein
DFR	Dihydroflavonol-4-reductase
dpi	Days post infiltration
dsRNA	Double-stranded RNA
EST	Expressed sequence tag
miRNA	Micro RNA
MP	Movement protein
PDS	Phytoene desaturase
PKS	Polyketide synthase
2PS	2-pyrone synthase
PTGS	Post transcriptional-gene silencing
RdDM	RNA-directed DNA methylation
RDR/RdRp	RNA-directed RNA polymerase
RISC	RNA-induced silencing complex
sgRNA	Subgenomic RNA
siRNA	Small interfering RNA
sRNA	Small RNA
ssRNA	Single-stranded RNA
tasiRNA	Trans-acting small interfering RNA
TF	Transcription factor
TRV	Tobacco rattle virus
VIGS	Virus-induced gene silencing
VSR	Viral suppressors of RNA silencing

Abstract

RNA silencing is a conserved mechanism that occurs in a broad range of eukaryotes, which is regulated by small RNAs (sRNAs). RNA silencing operates to control gene expression and maintain genome integrity. Virus-induced gene silencing (VIGS) in plants is a natural antivirus mechanism that has adapted from the general RNA silencing system. To counter the antivirus RNA silencing, plant viruses have evolved to encode viral suppressors of RNA silencing (VSRs). Nowadays VIGS is usually referred to as the technology that uses recombinant viruses to knock down the expression of plant endogenous genes. *Gerbera hybrida* (gerbera) is a model species in the family of Asteraceae. As a highly heterozygous species, gerbera lacks efficient functional genetic approaches other than gene transfer. The aim of the present study was to develop a *Tobacco rattle virus* (TRV, genus *Tobravirus*) induced gene silencing system for gerbera, and use TRV VIGS to characterize functions of chalcone synthase (CHS) encoding genes in the plant.

Preliminary VIGS experiments on the cultivar Terraregina, by syringe infiltration and applying previously developed TRV vectors, did not result in visible VIGS phenotypes due to the absent of TRV RNA2 in the up non-infiltrated leaves. Consequently, I first aimed to study the mechanism of TRV VIGS, and tried to develop new VIGS vectors based on TRV RNA1.

I investigated the role of two important TRV proteins of the 16K VSR and the 29K movement protein (MP) on TRV infection and TRV VIGS, and developed TRV RNA1 based VIGS vectors. For accomplishing this, a series of TRV RNA1 mutants have been constructed to disrupt the 16K, or to replace its 29K with *Tobacco mosaic virus* (TMV, genus *Tobamovirus*) 30K MP. TRV RNA1 vector, carrying a fragment of the gene encoding *Nicotiana benthamiana* PDS to replace part of the *16K* sequence, induced *PDS* gene silencing systemically in *N. benthamiana*. However, this has found to be less efficiently than the original TRV VIGS system when the wild-type RNA1 and RNA2:PDS were used. The infection experiments demonstrated that 16K was required for TRV long distance movement, and helped in maintaining the integrity of the TRV RNA2 genome. In addition, TRV 29K alone did not suppress RNA silencing in the co-infiltration assay, but it could suppress RNA silencing in the context of RNA1 replication. TRV 29K may be the first VSR whose silencing suppression functions are found to be directly linked to viral replication.

The original TRV vector system was finally adopted for VIGS in gerbera. TRV VIGS was optimized for gerbera by screening for TRV sensitive cultivars and by improving its inoculation methods. Intensive gene silencing phenotypes were achieved both in green tissues and in floral tissues,

demonstrated by knocking down genes involved in isoprenoid biosynthesis (phytoene desaturase: *GPDS*; H and I subunits of Mg-chelatase: *GChl-H* and *GChl-I*), flower pigmentation (*chalcone synthase*: *GCHS1*), and flower development (GLOBOSA-like MADS domain transcription factor: *GGLO1*). Unexpectedly, a gerbera polyketide synthase encoding gene, *G2PS1*, that has no apparent connections to the carotenoid or chlorophyll biosynthesis, was knocked down by the photo-bleaching that was induced by the silencing of *GPDS*, *GChl-H* and *GChl-I*, or by the herbicide norflurazon. We have demonstrated for the first time that the using of VIGS in an Asteraceaeous species. Our data also suggested that the selection and use of a marker gene for VIGS should be strictly evaluated.

A new CHS encoding gene, *GCHS4*, was characterized in gerbera. Together with the two previously identified *GCHS1* and *GCHS3*, gerbera CHSs are represented by a three-gene family. Each gerbera *CHS* shows a distinct expression pattern. *GCHS3* is particularly expressed in gerbera pappus. In partnership with the concomitantly expressed *GCHS1*, they are involved in the biosynthesis of colorless flavonoids. *GCHS4* is the only *CHS* that is naturally expressed in the leaf petiole and inflorescence scape, and it is responsible for cyanidin biosynthesis in those tissues. *GCHS4* is also the only *CHS* that was induced by environmental stresses in the leaf blade. Both *GCHS1* and *GCHS4* are markedly expressed in gerbera petals, and *GCHS4* mRNA actually takes the majority of *CHS* mRNAs in the later stages of petal development. Nonetheless, VIGS experiments, by target silencing *GCHS1* or *GCHS4* independently, demonstrated that GCHS1 is the predominant functional CHS in gerbera petals. Thus, *GCHS4* in gerbera petals seems to be regulated post-transcriptionally.

In conclusion, the results of this study shed new light on the mechanism of TRV VIGS.

The established TRV VIGS system provides a valuable tool for functional genomics in gerbera.

1. INTRODUCTION

1.1 *Gerbera hybrida* ─ a model for Asteraceae

Gerbera hybrida (gerbera) is one of the most important ornamental species in the world. According to the International Trade Center, the total sale for gerbera cut flowers was €123.5 million at 2011, which ranks 4th in cut flowers sales after rose, chrysanthemum, and tulip in Dutch auctions (Plasmeijer & Yanaim, 2012). Gerbera, also known as African daisy, is named after a German medical doctor, Traugott Gerber (Ambrosius, 2003). The genus of *Gerbera* has up to 70 species that can be found in South Africa, Asia, and South America (Ambrosius, 2003). *Gerbera jamesonii* (Fig. 1A and 1C) originates from the Barberton area of South Africa, and is the closest wild relative of *Gerbera hybrida*. Some other wild species, such as *Gerbera viridifolia* (Fig. 1B), have also been used in cross-breeding with *Gerbera jamesonii* (Hansen, 1999). Nowadays, most commercial cultivars are thought to have arisen from these crosses. Started first at the end of 19th century in Cambridge, England, gerbera breeding has been most active in the Netherlands, Denmark, Germany, Israel, Japan, and United States (Rogers and Tjia 1990).

Fig. 1. *Gerbera hybrida* is a hybrid between two native South African species: *Gerbera jamesonii* (A) and *Gerbera viridifolia* (B). C. The first color illustration of the Barberton Daisy (*Gerbera jamesonii*). Sources of image: A, http://aylestone8.wordpress.com-/tag/gerberaviridifolia/; B, http://www.gerbera.org/; C, Penningsfeld & Forchthammer, 1980.

Gerbera is a member of the Asteraceae family, which is one of the largest families among flowering plants. This family also comprises many other economically important species, such as sunflower (*Helianthus annuus*), lettuce (*Lactuca sativa*), chicory (*Cichorium intybus*), marigold

(*Calendula officinalis*), and chrysanthemum (*Dendranthema*) (Judd *et al*., 2002). As a typical Asteraceae, gerbera has a complex inflorescence (capitulum) that bears hundreds of flowers that are morphologically different. The whole capitulum has the appearance and function similar as a single flower, and it is composed of three types of flowers. The marginal "ray" flowers are female and highly zygomorphic, with a relatively large outer corolla lip and two rudimentary corolla internal petals, which form 1-3 whorls of petal-like structures on the capitulum. "Disc" flowers are small, more radially symmetrical, and hermaphrodite. In many cultivars, between the outer ray flowers and central disc flowers there are "trans" flowers, which are also female like ray flowers, but with much shorter ligules (Kloos *et al*., 2004; Teeri *et al*., 2006a,b). In addition, gerbera varieties show a wide spectrum of color patterns, and harbor Asteraceae-specific secondary metabolites, including flavonoids in addition to polyketide derived metabolites as defence compounds against fungal diseases and insects (Koskela *et al*., 2011). All those characteristics make gerbera a unique model for studying flower development and secondary metabolites (Teeri *et al*., 2006a,b).

Two decades of studies in our laboratory have already made *Gerbera hybrida* an Asteraceae model for research of flower development and secondary metabolite biosynthesis (Elomaa *et al*., 1993; Helariutta *et al*., 1996; Eckermann *et al*., 1998; Yu *et al*., 1999; Kotilainen *et al*., 2000; Uimari *et al*., 2004; Laitinen *et al*, 2005; Broholm *et al*., 2008; Koskela *et al*., 2011; Tähtiharju *et al*., 2012). Gerbera is a diploid plant, with an estimated genome size of 2500 Mb (Bennett and Leitch 1997). The gerbera expressed sequence tag (EST) database now contains more than 300 000 EST sequences, and the ongoing large-scale sequencing efforts on gerbera will increasingly provide us with more fundamental genomic information in addition to candidate genes putatively involved in the processes that we are interested in (Teemu Teeri and Paula Elomaa, unpublished data). Thus, there is a great demand for efficient approaches to identify functions of corresponding genes.

Gerbera hybrida is highly heterozygous, and suffers from strong inbreeding depression. Functional studies have to be done by reverse genetic approaches through producing stable transgenic lines by using *Agrobacterium tumefaciens*-mediated gene transfer, or by particle bombardment, which are labor-intensive and time-consuming (Teeri, *et al*., 2006a, b; Elomaa and Teeri, 2001; Elomaa *et al*., 1993). Virus-induced gene silencing (VIGS) is a recently developed gene knock down technique for identifying gene functions in plants. It offers an attractive alternative, as it allows for rapid preliminary identification of gene functions without stable plant transformation (Burch-Smith *et al*., 2004).

1.2 RNA silencing

1.2.1 The history of RNA silencing

RNA silencing was first discovered in plants but was subsequently found to occur widely in most eukaryotic organisms. It is a genetically conserved process that is regulated by small RNAs (sRNA) and plays essential roles in gene regulation, development control, genome defense and adaptive responses to both biotic and abiotic stresses (Baulcombe, 2004; Gunter & Tuschl, 2004 ; Li & Ding 2006; Brodersen & Voinnet, 2006). The importance and potential use of RNA silencing have been emphasized recently by the 2006 Nobel Prize in Physiology or Medicine, which was awarded to Andrew Z. Fire and Craig C. Mello for their discovery of RNA interference in *Caenorhabditis elegans* (Fire *et al*., 1998).

RNA silencing as a scientific topic started to attract general interest about two decades ago (Dougherty & Parks, 1995; Baulcombe, 2000; Carrington, 2000). However, the first report on RNA silencing could be traced back to year 1928 when some tobacco plants infected with *Tobacco ringspot virus* (TRSV), the upper non-inoculated leaves got recovered, and was resistance to a secondary infection (Baulcombe, 2004). Later, an *Agrobacterium*-mediated double transformation was done by Matzke *et al*. (1989) who reported that a T-DNA insert was inactivated by the introduction of a second T-DNA. Those authors suggested that the homologous sequence shared by the promoters in the two T-DNA vectors have caused methylation of the promoter sequence. In the following year (1990), during the processes of developing transgenic plants, it was reported that the introduced sense transgenes eventually silenced themselves, and, in some cases, the homologous endogenous genes were also silenced (Napoli *et al*., 1990; van der Krol *et al*., 1990). In those studies, the aim was to enhance pigmentation in *Petunia hybrida* (petunia), for which two enzymes in the flavonoid biosynthesis pathway, dihydroflavonol-4-reductase (DFR) and chalcone synthase (CHS), were overexpressed. Unexpectedly, flowers of the transgenic plants manifested varied pigmentation levels, including deep purple, patters of purple and white, and pure white. Analysis of gene expression in the transformed populations revealed that in some lines both the introduced and endogenous forms of the *CHS* or *DFR* were “turned off”, or silenced to varying degrees (Napoli *et al*., 1990; van der Krol *et al*., 1990). Similarly, the overexpression of a truncated polygalacturonase gene in tomato caused a strong reduction of the endogenous homologous gene during fruit ripening (Smith *et al*., 1990). During the pre-genomic era, this phenomena, gene suppression at the RNA level, was described as co-

suppression or post transcriptional gene silencing (PTGS) (Baulcombe, 1996; Chen, 2010).

In plant virus studies, PTGS was described as cross-protection for some time. Tobacco plants, transformed with *Tobacco etch virus* (TEV) coat protein (CP), were resistant to a secondary infection of TEV, but susceptible to unrelated *Potato virus X* (PVX) (Lindbo *et al*., 1993). Later Baulcombe's group (Ratcliff *et al*., 1997) demonstrated that *Nicotiana clevelandii* plants, infected with *Tomato black ring nepovirus* (strain W22), were susceptible to unrelated PVX and displayed increased virus-related symptoms, but were resistant with a modified PVX that carried a W22 fragment. It was noticed at that time that sequence similarity between the transformed gene and the infected virus, or the primary and secondary infected virus, was required for the induction of cross-protection. In addition, it was observed that the transformed TEV CP was actually actively transcribed, but the corresponding mRNA failed to accumulate. Thus, as early as in 1990s, the co-suppression or cross-protection was proposed to be localized in the cytoplasm and occurring at the post transcriptional level.

Thereafter, several other studies have shed new light on the underlying mechanism of PTGS. In the search to find out the reason that caused *CHS* silencing in the transgenic petunia, Stam *et al.* (1997) found that the multiple T-DNA copies in the same locus were in an inverted-repeat (IR) orientation. More directly, Waterhouse *et al.* (1998) proved that the formation of double-stranded RNA (dsRNA) was the inducer of cross-protection to *Potato virus Y* (PVY) infection. Two transgenic tobacco lines that contained either a sense or antisense open reading frame (ORF) of PVY were susceptible to PVY infection. However, when the sense and antisense gene were brought together by crossing, the progeny lines that contained both the sense and anti-sense gene were found to be highly resistant to PVY infection. Waterhouse and colleagues also found that the introduction of an IR form of truncated GUS was more efficient at silencing the host expressed GUS in rice (*Oryza sativa*) than the transformation of either sense or antisense GUS fragment. Similar discoveries were also reported in nematodes (Fire *et al*., 1998), protozoa (Ngo *et al*., 1998), and insects (Kennerdell & Carthew, 1998).

A major breakthrough in RNA silencing was the discovery of small RNAs (sRNA). In the screen of sRNA species in plants that underwent PTGS, Hamilton and Baulcombe (1999) found that sRNAs (approximately 25 nucleotides in size) were associated with PTGS. From then on, the mechanism of RNA silencing pathway started to take shape. First, RNA silencing requires the formation of the dsRNA, by the introduction of foreign nucleotide sequences through T-DNA transformation or by infection of a plant virus. This dsRNA is then processed by Dicer-like (DCL)

endonucleases into sRNAs, which subsequently guide sequence specific RNA degradation.

1.2.2 RNA silencing pathways

Since the first discovery of RNA silencing in plants in the early 1990s (Matzke *et al*., 1989; Linn *et al*., 1990; Napoli *et al*., 1990; Smith *et al*., 1990; van der Krol *et al*., 1990), remarkable progress has been made in the understanding of the molecular mechanisms that drive RNA silencing. In plants, sRNAs of mainly of 21- to 24-nucleotides (nt) in size, are the inducers of RNA silencing. Despite the diverse functions of RNA silencing and the biogenesis of sRNAs, all RNA silencing pathways share four consensus biochemical steps (Ruiz-Ferrer & Voinnet, 2009): 1) formation of dsRNA; 2) procession of dsRNA into sRNAs; 3) stabilization (by 2'-*O*-methylation) and exportation (from cell nucleus to cell cytosol) of sRNAs; 4) formation of the RNA-induced silencing complex (RISC) and its directed target (DNA or RNA) suppression. A number of enzymes are now known to be involved in the RNA silencing processes. For the production of sRNAs, the plants use a series of dsRNA specific RNase III-type Dicer enzymes (Xie *et al*., 2004). The 3' overhanging ends of sRNAs are methylated by methyltransferase HUA ENHANCER 1 (HEN1) (Yu *et al*., 2005), which protects them from polyuridylation and degradation. The stabilized sRNA duplexes may stay in the nucleus for chromatin-level activities, or they may be exported to cytosol, probably via the exportin-5 homolog HASTY (HST), for PTGS (Poulsen *et al*., 2013). One strand of sRNA duplex, the guide strand, combines with ARGONAUTE (AGO) to form RISC, which directs the sequence-specific suppression. The other strand, passenger strand, is degraded (Poulsen *et al*., 2013). The arabidposis (*Arabidopsis thaliana*) genome encodes 4 DCL and 10 AGO proteins. Two other protein families, RNA-directed RNA polymerase (RDR) and double-stranded RNA-binding domain (dsRBD), have also been shown to work together with DCL and AGO. There are a total of 6 RDRs and 5 dsRDBs in arabidopsis (Eamens *et al*., 2008).

Genome-wide profiling of sRNAs in arabidopsis revealed that the most abundant sRNA species are 24-nt small interfering RNAs (siRNAs) (Xie *et al*., 2004; Gustafson, *et al*., 2005; Lu *et al*., 2005; Kasschau *et al*., 2007; Voinnet, 2009), which are derived mostly from transposable elements and other repetitive sequences, and act to silence such loci at the transcriptional level through RNA-directed DNA methylation (RdDM) and repressive chromatin modifications (Poulsen *et al*., 2013). This pathway (Fig. 2A) utilizes both plant specific DNA-dependent RNA polymerase pol IV and pol V (Wierzbicki *et al*., 2008; Zhang *et al*., 2008), and RDR2, which copy single-stranded RNA (ssRNA) into dsRNA. These dsRNA molecules

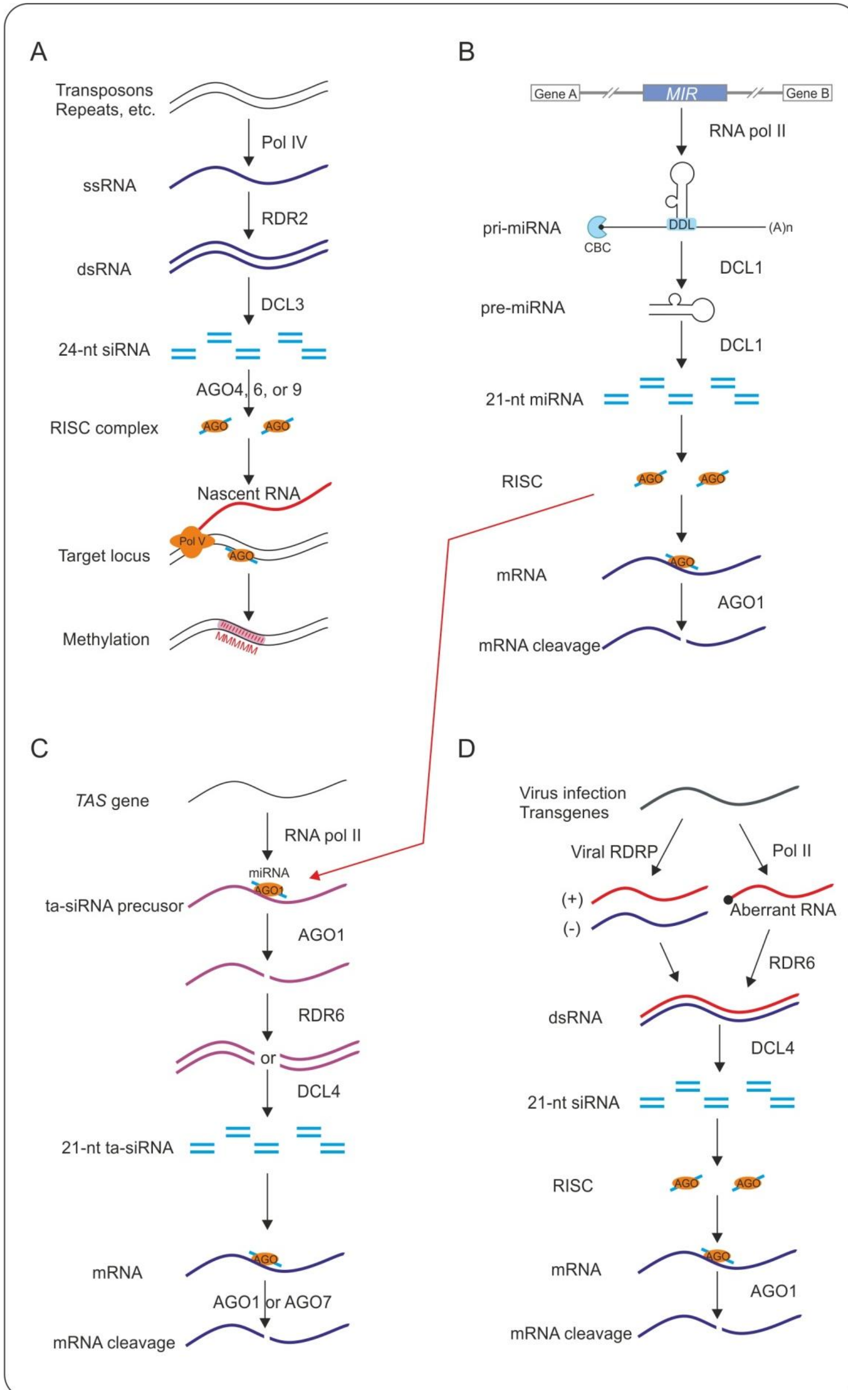
A
Transposons
Repeats, etc.
Pol IV
ssRNA
RDR2
dsRNA
DCL3
24-nt siRNA
AGO4, 6, or 9
RISC complex
AGO
Nascent RNA
Pol V
Target locus
Methylation
MMMMM
B
Gene A
MIR
Gene B
RNA pol II
pri-miRNA
CBC
DDL
(A)n
DCL1
pre-miRNA
DCL1
21-nt miRNA
RISC
mRNA
AGO1
mRNA cleavage
C
TAS gene
RNA pol II
miRNA
AGO1
ta-siRNA precusor
AGO1
RDR6
or
DCL4
21-nt ta-siRNA
mRNA
AGO1 or AGO7
mRNA cleavage
D
Virus infection
Transgenes
Viral RDRP
Pol II
(+)
(-)
Aberrant RNA
RDR6
dsRNA
DCL4
21-nt siRNA
RISC
mRNA
AGO1
mRNA cleavage

Fig. 2. Simplified RNA silencing pathways in plants. A, Silencing targeted to transposable elements and other repetitive sequences is caused by the RNA-dependent DNA methylation directed by 24-nt siRNAs, which are produced with the action of DCL3. Host Pol IV, Pol V, RDR2, and AGOs (Ago4/6/9) are involved. B, MicroRNA (miRNA) directed gene silencing. miRNAs are 21-nt sRNAs, which are produced from endogenous *MIR* genes, with the action of RNA pol II and DCL1. Host AGO1 is involved in the miRNA directed silencing. C, Trans-acting siRNA (ta-siRNA) targeted RNA silencing. ta-siRNAs are 21-nt sRNAs, which are produced from the endogenous *TAS* gene with the action of DCL4. The production of ta-siRNA is miRNA pathway dependent. Host proteins of RNA pol II, RDR6, and AGOs (AGO1/7) are involved. D, Exogenous siRNA directed RNA silencing. Exogenous siRNAs are 21-nt siRNAs, derived from the directly introduced dsRNAs, transgenes, or infected viruses. Host proteins of RNA pol II, RDR6, AGO1, and DCL4 are involved. This diagram is modified from the following sources: Brodersen & Voinnet, 2006; Vaucheret, 2006; Voinnet, 2009; Ghildiyal & Zamore, 2009; Chen, 2009; Simon & Meyers, 2011.

are then cleaved by DCL3 into 24-nt siRNAs that are recruited by the RISC containing AGO4, AGO6, or AGO9 to guide the chromatin modifications to the homologous DNA sequence (Zilberman *et al*., 2003; Zheng *et al*., 2007; Havecker *et al*., 2010).

The second most abundant sRNAs are microRNAs (miRNAs). miRNAs are endogenous sRNAs that originate from imperfect fold-back stem-loop RNAs (pri-miRNAs) that are transcribed from *MIR* genes by RNA polymerase II (Pol II) (Lee *et al*., 2004). *MIR* genes are non-coding sequences that are located between the protein-coding genes (Voinnet, 2009) (Fig. 2B). Procession of the pri-miRNAs yields pre-miRNAs, which are further cleaved into small RNA duplexes (Fig. 2B). DCL1 is the enzyme that makes both these processions within the miRNA precursors through the interaction with a dsRNA binding protein HYPONASTIC LEAVES1 (HYL1) (Kim, 2005). In plants, miRNA duplexes are typically 21-nt in size, with two-nucleotide overhangs at the 3'-ends. Mature miRNAs are methylated by HEN1, and combined with AGO1 to repress their target mRNAs by translational inhibition, accelerated mRNA decay, or slicing within miRNA-mRNA base paring sequences (Llave *et al*., 2002; Eulaio *et al*., 2008). Most of known plant miRNAs target transcription factors that regulate crucial steps during plant development (Rhoades *et al*., 2002; Flynt & Lai, 2008; Garcia, 2008). Some miRNAs regulate other biological functions, including hormonal control, immune responses, and adaptation to biotic and abiotic stresses (Bartel, 2004; Fujii *et al*., 2005; Sunkar *et al*., 2007; David, 2008; Voinnet 2008).

The third class of sRNAs are *trans*-acting siRNAs (ta-siRNAs). The ta-siRNAs are derived from non-coding sequences of *TAS* genes in the genome that serve as the precursors of ta-siRNAs. The synthesis of ta-siRNA is miRNA pathway dependent, and starts from the miRNA

processed single stranded *TAS* gene transcripts (Peragine *et al*. 2004; Vazquez *et al*. 2004a; Allen *et al*. 2005). RDR6 associates with dsRNA binding protein DRB4, and copies one of the two miRNA cleaved *TAS* transcripts into dsRNAs (Peragine *et al*. 2004; Vazquez *et al*. 2004a,b; Allen *et al*. 2005). These dsRNAs are further processed by DCL4 into 21-nt ta-siRNA duplexes (Hiraguri *et al*. 2005; Adenot *et al*. 2006). The ta-siRNAs suppress their targets in *trans* by guiding mRNA cleavage, in the same manner as for miRNA (Fig. 2C). AGO1 is involved in most ta-siRNA directed regulations (Baumberger & Baulcombe, 2005; Qi *et al*., 2005), but AGO7 seemed to be involved in the TAS3-mediated regulation (Peragine *et al*., 2004; Allen *et al*., 2005; Adenot *et al*., 2006).

Another important class sRNAs are exogenous siRNAs. Exogenous triggers, such as directly introduced dsRNA, transgenes, and infected viruses are well-known sources of exogenous siRNAs. RDR6 is required for sense transccripts triggered PTGS (Dalmay *et al*., 2000; Mourrain *et al*., 2000). It was thought that RDR6 can recognize and use as templates certain transgene transcripts with aberrant features of, e.g. lack of 5'-cap, and convert those single stranded aberrant RNAs into dsRNAs (Gazzani *et al*., 2004). For infected viruses, dsRNAs are intermediates of virus replication that are formed by the action of RNA-dependent RNA polymerase (RDR)-catalyzed synthesis. Viral dsRNA can also be formed by self-annealing of complementary regions within single-stranded viral RNA (Fig. 2D). DCL4 and AGO1 are the main contributors to the exogenous siRNA induced silencing pathway, and the majority siRNAs are 21-nt in length (Ding & Voinnet, 2007; Wang *et al*., 2011).

In addition to the four major classes of sRNAs addressed above, there are many other sRNAs that have been discovered in plants. Natural antisense siRNAs (nat-siRNAs) are derived from two mRNAs that harbor complementary regions (Borsani *et al*., 2005; Katiyar-Agarwal *et al*., 2006; Held *et al*., 2008). The arabidopsis genome encodes more than 2000 *cis*-antisense gene pairs (Borsani *et al*., 2005). dsRNAs can be formed between transcripts of *cis*-antisense gene pairs that are located nearby each other. sRNAs longer than miRNAs and siRNAs have also been found in arabidopsis recently (Katiyar-Agarwal, *et al*., 2007). Their functions on gene regulation remain to be elucidated.

To summarize, sRNAs are repressors of gene expression, and the dsRNAs are the inducers of RNA silencing. The sources of both endogenous and exogenous siRNAs, such as transposons, viruses and transgenes, are also the targets of RNA silencing. In contrast, miRNA and ta-siRNA target genes are distinct from their source *MIR* and *TAS* genes (Bartel, 2004).

1.3 Virus-induced gene silencing (VIGS)

Viruses are one of the most destructive plant pathogens, like bacteria and fungi. Plant viruses, however, are intracellular pathogens and with genomes that replicat within the host cells. Hence, RNA silencing, or VIGS, plays an essential role in anti-viral defense.

VIGS is a natural viral immunity mechanism of plants. Virus Infections are always coupled with the accumulation of viral siRNAs, which in turn combine with AGO proteins and guide the cleavage of viral RNAs (Ruiz-Ferrer & Voinnet, 2009; Voinnet, 2005). Therefore, viruses are both the inducers and also the targets of VIGS. Viral siRNAs are derived from several sources. One main source is the hybrid dsRNAs, which are formed by the annealing of positive and negative single-stranded RNAs of viral replication intermediates. The second source is provided by the internal hairpin-loop structures formed within the single-stranded viral RNA (Voinnet, 2005; Molnár *et al*., 2005). In addition, host RDR1, RDR2 and RDR6 are involved in some siRNA synthesis (Wang *et al*., 2010). This biogenesis pathway resembles the host endogenous siRNA pathway. Aberrant viral single-stranded RNAs that lack quality control markers are templates for RDRs mediated secondary siRNA production (Wang *et al*., 2010).

Most viral siRNAs are 21- and 22-nt siRNAs that are processed by DCL4 and DCL2, respectively (Ding & Voinnet, 2007). DCL4 plays a more important role than DCL1 in combating RNA virus infections in plants. The 21-nt siRNAs constitude 72-86% of the total viral siRNAs, which are more abundant than those of 22-nt (10-21%) (Wang *et al*., 2010). DCL3 plays a minor role, which is indicated by the low levels of 24-nt siRNAs that are associated with the RNA virus infection. AGO1 is the most dominant AGO protein for the siRNA-directed antiviral defense. AGO2 and AGO7 are also involved in the pathway (Qu *et al*., 2008; Harvey *et al*., 2011; Jaubert *et al*., 2011; Wang *et al*., 2011). In contrast, plants infected with DNA viruses, accumulate all three kinds of siRNAs, with 24-nt siRNAs sharing the largest proportion (Akbergenov *et al*., 2006; Blevins *et al*., 2006; Moissiard & Voinnet, 2006). Thus, DCL3 plays a major role in plant resistance to DNA viruses. Profiling of germinivirus siRNAs showed that the 21- and 22-nt siRNAs had originated from the coding regions of the virus genome, whereas the 24-nt siRNAs were mostly derived from the intergenic regions of the genome (Rodríguez-Negrete *et al*., 2009; Yadav & Chattopadhyay, 2011). These 24-nt siRNAs are thought to be involved in the methylation of the genome’s intergenic regions by the RdDM pathway, which is important for plant resistance to DNA virus infection.

1.3.1 Viral suppressors of RNA silencing

To counter the host antiviral silencing, plant viruses have evolved to contain viral suppressors of RNA silencing (VSR). Every plant virus that has been screened thus far contains at least one VSR (Li and Ding 2006), which suggests a necessary and universal counterstrategy. A single virus, such as *Citrus tristeza virus* (CTV), *Potato virus A* (PVA), and germiliviruses, may contain multiple VSRs (Brigneti *et al*., 1998; Lu *et al*., 2004; Vanitharani *et al*., 2004; Rajamäki & Valkonen, 2010).

Most VSRs are multifunctional proteins. Apart from being VSRs, they also have essential roles as coat proteins (CP), replicases, movement proteins (MP), helper components for viral transmission, proteases or transcriptional regulators. VSRs encoded by viruses from different families share no similarities at amino acid sequence level. However, in the case of viruses belonging to the same family, the VSRs are mostly homologs and locate at the same place in the viral genomes, regardless of their sequence similarities (Li *et al*., 1999; Li *et al*., 2004; Te *et al*., 2005).

VSRs suppress RNA silencing through diverse actions that may target various steps throughout of RNA silencing (Fig. 3). The first mode of action by VSRs is to bind to long dsRNAs, which inhibits siRNA biogenesis. P14 of *Pothos latent aureusvirus* and P38 of *Turnip crinkle virus* (TCV) have been shown to have the ability to bind dsRNA in a size dependent way (Merai *et al*., 2005, 2006). A more common mechanism of VSRs is sequestering siRNA to prevent RISC assembly. P19 of tombusviruses, the best characterized VSR thus far, prevents RNA silencing by siRNAs binding with a high affinity (Silhavy *et al*., 2002). The P38 and the 2b of *Tomato aspermy cucumovirus* also have siRNA binding ability, but their mechanisms of siRNA binding share no similarities with those of tombusviruses P19s (Chao *et al*., 2002; Chen *et al*., 2008). Some siRNA binding VSRs, such as P19 of *Carnation Italian ringspot virus*, HC-Pro of *Tobacco etch virus* (TEV), and P122/P130 of *Tobmovirus*, function through compromising the step of 2'-O methylation (Ebhardt *et al*., 2005; Yu *et al*., 2006; Csorba *et al*., 2007; Vogler *et al*., 2007; Lozsa *et al*., 2008), which is a key step in the biosynthesis of si/miRNA and assembly of RISC. VSRs can also interact directly or indirectly with RISC components, such as AGO proteins, to inhibit RNA silencing. The 2b protein of *Cucumber mosaic virus* (CMV) can physically interact with the PAZ domain and part of the PIWI domain of AGO1, to prevent its slicing ability (Zhang *et al*., 2006). In contrast, P0 of the phloem-limited poleroviruses do not interact with AGO1 directly, but accelerate AGO1 degradation through interacting with the SCF family of E3-ligase S-phase kinase-related protein-1 components. A number of VSRs, including P38 and P1 of *Sweet potato mild mottle ipomovirus*, interact with AGO1 through their evolved AGO hook motifs, WG/GW repetitive motifs, required for host proteins of AGO binding

(Azevedo *et al*., 2010; Giner *et al*., 2010). Another known mode of action by VSRs is to target the amplification of antiviral silencing. The amplified secondary siRNAs are known to play an essential role in plant resistance to RNA virus (Ruiz-Ferrer and Voinnet 2009; Garcia-Ruiz *et al*., 2010; Wang *et al*., 2010). The 2b and V2 of *Tomato yellow leaf curl virus* are typical members in this type. They inhibit RNA silencing by interacting with host RDRs, which are important components for secondary siRNA biogenesis (Diaz-Pendon *et al*., 2007; Zrachya *et al*., 2007; Glick *et al*., 2008).

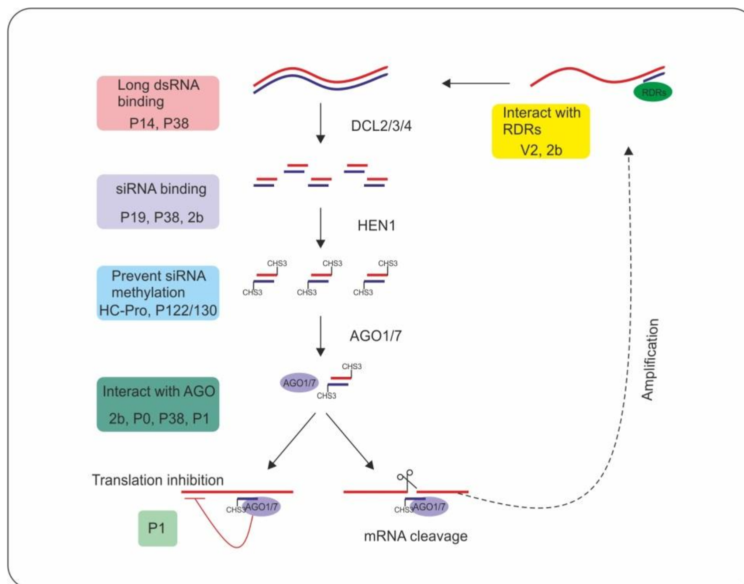

Fig. 3. Antiviral RNA silencing in plants and its suppression by viral suppressors of RNA silencing (VSR). RNA silencing starts by the recognition of viral dsRNAs, which are processed by Dicer-like endonucleases (DCL) into small interfering RNAs (siRNA). The siRNAs are then methylated by HUA ENHANCER 1 (HEN1), and loaded onto ARGONAUTE (AGO) to form RNA-induced silencing complex (RISC). Afterwards, RISC targets the viral RNAs by translation arrest or by slicing. Secondary siRNAs are produced in an amplification loop through the actions of RNA-directed RNA polymerase (RDR). VSRs encoded by different viruses can suppress virus-induced gene silencing by targeting different steps of the process, thereby preventing the assembly of different effectors or inhibiting their actions. Target points of VSRs are indicated by the colored boxes. This diagram is modified from the source: Burgyán & Havelda, 2011.

To inhibit the RNA silencing that targets to DNA virus infection, VSRs encoded by DNA viruses such as the AL2 of *Tomato golden mosaic virus* (TGMV) and L2 of *Beet curly top virus* (BCTV), can prevent transcriptional

gene silencing in plants by reducing DNA methylation (Wang *et al*., 2005; Bisaro *et al*., 2006).

The molecular base of VSRs to suppress RNA silencing may be more complicated than we understand so far. Indeed, P38 of TCV uses multiple modes of action for RNA silencing suppression, and has the abilities to bind to long dsRNAs and siRNA duplexes, and to interact with AGO1 (Azevedo *et al*., 2010). Similarly, P19 of tombusviruses inhibits AGO1 translation through the enhanced miR168 expression, in addition to binding siRNA duplexes (Várallyay *et al*., 2010). It is possible that many other VSRs also act with complex modes to combat antiviral RNA silencing, but this remains to be elucidated.

1.3.2 VIGS as a functional genomics approach

The term of VIGS was first proposed by A. van Kammen (1997) to describe the phenomenon of recovery from virus infection. Nowadays, VIGS is usually referred to as a technique that utilizes recombinant virus to specifically reduce endogenous gene expression. This technique uses the plant's natural antivirus RNA silencing mechanisms, which targets to the virus carried host gene fragment in the recombinant virus genome, to knock down the expression of homologous endogenous genes (Kumagai *et al*., 1995).

Provided that a suitable viral vector is available, performing VIGS mainly involves three steps, which are: to clone the target fragments of the host genes into the VIGS vector, to infect the host plants, and to interpret the VIGS phenotypes (Fig. 4). Compared with other conventional approaches for functional analysis, VIGS has many significant advantages (Burch-Smith *et al*., 2004; Senthil-Kumar and Mysore 2011c). VIGS is easy and rapid. It often involves cloning and agro-inoculation, and normally takes within a month from infection to the manifestation of silencing phenotypes. VIGS excludes the production of stable transgenic plants, which is extremely challenging in many economically important plant species (Brigneti *et al*., 2004). In addition, only partial information of a gene sequence is necessary to silence a gene. VIGS is convenient for silencing either a single member of a gene family or all family members at a time by using gene specific or highly conserved sequences. With the ease of large-scale sequencing, VIGS is particularly useful for species that are recalcitrant to stable genetic transformation but whose functional studies are essentially needed.

VIGS has been utilized in more than 30 plant species so far (Becker & Lange, 2010). Most of those species are from the Solanaceae family, such as species of *Nicotiana*, tomato (*Solanum lycopersicum*), and petunia

(*Petunia hybrida*), due to their high sensitivity to most of plant viruses (Brigneti *et al*., 2004; Chen *et al*., 2004,2005; Sahu *et al*., 2012). VIGS is also effective in many other important species, such as arabidopsis (Burch-Smith *et al*., 2006; Wang *et al*., 2006), soybean (*Glycine max*) (Zhang *et al*., 2010), pea (*Pisum sativum*) (Constantin *et al*., 2004), and cassava (*Manihot esculenta*) (Fofana *et al*., 2004). With the development of VIGS vectors that are naturally infectious to monocot species, VIGS has also been expanded to rice (*Oryza sativa*), barley (*Hordeum vulgare*), wheat (*Triticum araraticum*), and maize (*Zea mays*) (Holzberg *et al*., 2002; Scofield *et al*., 2005; Tai *et al*., 2005; Ding *et al*., 2006). Nowadays VIGS is mostly used for revealing gene functions in resistance to biotic and abiotic stresses, and in plant development. This has been extensively reviewed by Purkayastha and Dasgupta (2009). The extension of VIGS usage to several species of Ranunculale (*Aguilegia*, *Eshscholzia*, *Papaver* and *Thalictrum*) (Kramer *et al*., 2007; Drea *et al*., 2007; Orashakova *et al*., 2009), early diverging lineages of the eudicots, and to some tree species that have long life cycles (Naylor *et al*., 2005; Jansson & Douglas, 2007; Jia *et al*., 2010), also helps biologists to understand the evolution of biodiversity (Di Stilio, 2011).

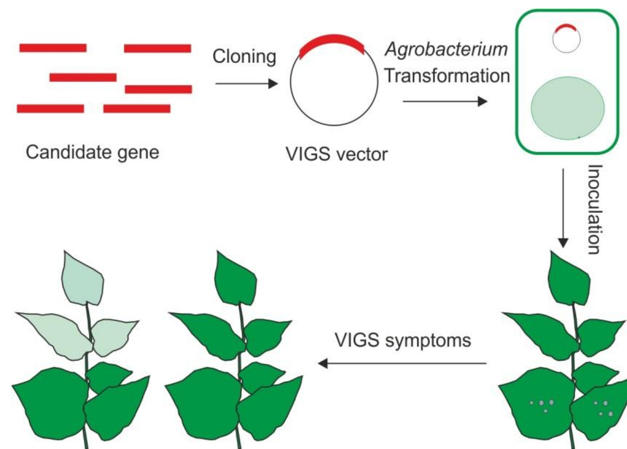

Fig. 4. Steps of virus-induced gene silencing (VIGS). VIGS starts by the cloning of the target gene fragment (200-1300 bp) into a virus infectious cDNA, which is in a binary vector under the control of the CaMV 35S promoter. The recombinant virus construct is then transformed into agrobacterium (*Agrobacterium tumefaciens*) for agrobacterium mediated virus infection. VIGS will target to the virus carried host gene fragment as to the viral genome, and also the endogenous host gene target.

Despite the improvements of using VIGS in many plant species, several limitations remain to be addressed. Theoretically, it is possible to develop any infecting viruses as vectors for VIGS in their host plants. However, there is no guarantee of high VIGS efficiency. Thus, for many plant species, the appropriate VIGS vectors are still absent. VIGS normally utilizes host gene fragments of between 200 to 1300 base pairs (bp) that target the middle regions of mRNAs (Liu & Page, 2008). Careful selection of the insert gene sequence should be made to avoid off-target silencing. Publicly available "siRNA-scan" softwares can be used to check potential off-target sequences in the databases (Xu *et al*., 2006; Senthil-Kumar & Mysore, 2011b). When possible, sequences that share perfect matches less than 11 bp with the predict off-target sequences should be selected. Another limitation is that VIGS mostly "knock down" but not "knock out" of a target gene. In many cases, the silencing is only transient, and the silencing effects are found in sectors. Thus, a large number of plants need to be treated for screening desirable phenotypes.

When the silencing of a target gene does not result in visible phenotypes, marker genes become useful for tracing the silencing in treated plants. Genes that encode phytoene desaturase (PDS), magnesium chelatase (CHL), and chalcone synthase (CHS) are the most widely used markers due to their visible silencing phenotypes (Kumagai *et al*., 1995; Chen *et al*., 2004; Igarashi *et al*., 2009). For example, co-silencing has been tested in petunia, whereby a *CHS* and a *R2R3-MYB* gene *EOBII* have been silenced simultaneously (Spitzer-Rimon *et al*., 2010). Green fluorescent protein (GFP) is another useful marker for VIGS in GFP transgenic plants (Quadrana *et al*., 2011). It is unlikely that the silencing of GFP in GFP transgenic plant will affect the expression of a target host gene. In addition, GFP expressed by a VIGS vector can also be a useful indicator for host gene silencing (Zhang *et al*., 2010).

1.4 Tobacco Rattle Virus

Tobacco rattle virus (TRV), together with *Pea early-browning virus* (PEBV) and *Pepper ringsport virus* (PepRSV), belong to the tobravirus genus (MacFarlane, 1999). The tobraviruse genomes contain two positive-sense single-stranded RNAs that are separately encapsidated into two rod-shaped particles (Fig. 5). The larger genome is RNA1. RNA1 of TRV is about 6.8 kb in size, and encodes 4 proteins. In the 5' proximal end, TRV RNA1 contains a large open reading frame (ORF) that encodes a 134-kDa protein of methyltransferase and helicase motifs. Readthrough translation of the stop codon of the *134K* gene produces a 194-kDa protein that contains the RNA-dependent RNA

polymerases (RdRp). Following the RdRp, TRV RNA1 encodes a 29-kDa movement protein (MP), and a 16-kDa cysteine-rich protein (CRP) that has been recently identified as a silencing suppressor (Ghazala *et al*. 2008; Martín-Hernández & Baulcombe, 2008; Martínez-Priego *et al*. 2008). The helicase and RdRp are translated directly from the RNA1 genome, while 29K MP and 16K proteins are translated from subgenomic RNAs (sgRNAs) 1a and 1b respectively. TRV RNA2 is smaller, with a size that varies considerably (1.8-3.9 kb) between different isolates, but always encodes a coat protein (CP). Some isolates may contain in their RNA2 genome one or both of the *2b* and *2c* genes, which are involved in nematode transmission (Angenet *et al*., 1986; Hernández *et al*., 1995; MacFarlane, 2010).

Geographically, TRV has been found throughout Europe, New Zealand, North America and Japan. As a plant pathogen, TRV has one of the widest host ranges among all the plant viruses. Natural infection has been reported in more than 100 plant species (Brunt *et al*., 1996). Inoculation with sap, plants of about 400 species in more than 50 families can be infected (Harrison & Robinson, 1978). TRV has continuously been a significant potato pathogen, which causes spraing or corky ringspot in potato tubers, which renders the crop unmarketable (MacFarlane, 2010). In addition, infection by TRV may cause a loss vigor and yield in tomato, tobacco, sugar beet, spinach, artichoke, celery, pepper and lettuce (Sudarshana & Berger, 1998).

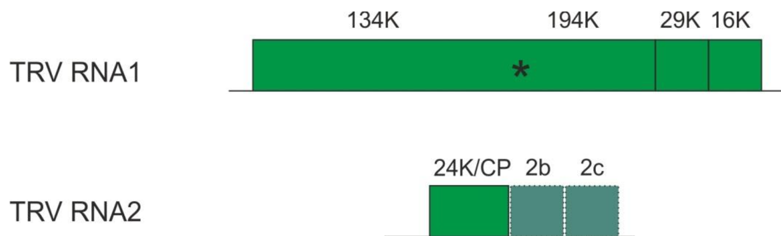

Fig. 5. Genome organization of tobacco rattle virus (TRV). TRV RNA1 encodes 4 proteins: 134K methyltransferase, 194K RNA-dependent RNA polymerase (RdRp), 29K movement protein, and 16K RNA silencing suppressor. The 194K protein is produced by reading through translation of the *134K* gene. TRV RNA2 encodes mainly a 24K coat protein. Some strains also contain one (strain TCM; Angenet *et al*., 1986) or both (strain PPK20; Hernández *et al*., 1995) of the 2b and 2c genes, which may be involved in nematode transmission.

TRV is primarily a soilborne pathogen transmitted by root-feeding nematodes. Seed transmission is possible in some plant species, such as

in *Solanum lycopersicum* and *Nicotiana benthamiana* (Senthil-Kumar & Mysore, 2011a). Typical of tobraviruses, TRV RNA1 can infect plants systemically in the absence of TRV RNA2. This kind of infection is referred to as non-multiplying (NM) infection in potato, and frequently occurs in vegetatively propagated crop plants (Liu *et al*., 2002). It is possibly caused by the limiting amount of inoculated virus particles, or by a resistance mechanism targeting the RNA2 genome (MacFarlane, 2010). The NM type infection usually spreads rapidly from cell to cell, but slower systemically. Consequently, its systemic symptoms develop slowly, but are more necrotic and persistent than those of M type infection (Infection with both RNA1 and RNA2) (Harrison & Robinson, 1978).

1.4.1 The role of the 16K protein

The *16K* gene is located at the 3' proximal of TRV RNA1, and encodes a CRP protein that is translated from the non-encapsidated sgRNA1b (Boccara *et al*., 1986). The 16K protein was detectable in protoplasts by western blotting (Angenent *et al*., 1989). Moreover, 16K was shown by immunogold electron microscopy to be localized predominantly in the nucleus (Liu *et al*., 1991). However, the 16K protein that is fused with red fluorescent protein (RFP) was found to be located primarily in the cytoplasm and, to a small amount, in the nucleus (Ghazala *et al*., 2008).

Functions of the 134/194K protein and 29K protein are already indicated by their sequences, which are homologous with viral replicase and movement proteins respectively. In contrast, the16K amino acid sequence offers few clues to its function. Initially, it was thought that 16K was involved in virus seed transmission because cysteine-rich proteins of other plant viruses such as the 12K protein of PEBV, a 16K homologue, has a role in seed transmission (Edwards, 1995; Wang *et al*., 1997). Several studies have been made to investigate the role of 16K protein, by making either mutations in *16K*, or replacing *16K* ORF with other viral genes. The results, however, have not been quite consistent.

The first study was done by Guilford *et al*. (1991), who developed two TRV *16K* mutants, one of which carried a partial deletion (73% of *16K* sequence) and the other a premature stop codon at the beginning in *16K*. Neither of these mutants showed significant difference in the infection of *Nicotiana tabacum* cv. Samsun NN compared to the wild type TRV. Thus, those authors concluded that neither the sequence nor the protein of 16K was essential for TRV replication or for cell to cell spread. When TMV CP was introduced into the TRV 16K deletion site, TMV CP protein was detectable with western blotting. The chimaeric-recombinant virus was able to replicate similar to that of the wild type TRV vector in the inoculated leaves, though it had a much lower long distance transport efficiency. More

recently, TRV *16K* mutants, with either partial deletion or frame shift mutation in the *16K* gene, was proved to be able to replicate in *Nicotiana benthamiana* and systemically spread to a similar extent as the wild type TRV (Martín-Hernández and Baulcombe 2008). Even so, the 16K mutations blocked the virus to enter apical meristems, which is consistent with the previous proposal that 16K mediates seed transmission (Wang *et al*., 1997).

A similar study was conducted by Liu and associates (2002). They showed that 16K was required for efficient virus replication and systemic movement. Those authors developed two TRV 16K mutants, by deletion of the entire *16K* gene or insertion of a premature stop codon at the beginning of the *16K* gene. Both of those mutants replicated poorly and were hardly detected in systemically infected leaves. This defect was rescuable, by the expression of 16K from TRV RNA2. Thus, they deduced that the 16K protein was a pathogenicity determinant, and perhaps a silencing suppressor.

TRV 16K, indeed, works as a RNA silencing suppressor. Direct evidence for this was obtained recently by three independent studies (Ghazala *et al*. 2008; Martín-Hernández and Baulcombe 2008; Martínez-Priego *et al*. 2008). Compared with P19 of *Tomato bushy stunt virus* (TBSV) and HCPro of *Tobacco etch virus* (TEV), TRV 16K is a relatively weak RNA silencing suppressor that targets to upstream steps of siRNA production (Ghazala *et al*., 2008).

1.4.2 TRV as VIGS vector

As reviewed recently (Becker & Lange, 2010; Senthil-Kumar & Kirankumar, 2011c), 22 RNA viruses and 12 DNA viruses have already been developed to serve as VIGS vectors for functional studies in more than 30 plant species. These numbers will continually increase with the need for extending VIGS to many other economically important species. Vectors based on TRV are stars among the numerous VIGS vectors, and have been used extensively in many plant species, such as *Nicotiana* species (Ryu *et al*., 2004; Senthil-Kumar *et al*., 2007), tomato (Fu *et al*., 2005; Liu *et al*., 2002), potato (*Solanum tuberrosum*; Brigneti *et al*., 2004), petunia (Chen *et al*., 2004; Spitzer *et al*., 2007), pepper (*Capsicum annum*; Chung *et al*., 2004), eggplant (*Solanum melongena*; Liu *et al*., 2012), deadly nightshade (*Solanum nigrum*; Hartl *et al*., 2008), Californian poppy (*Eschscholzia californica*; Wege *et al*., 2007), opium poppy (*Papaver somniferum*; Hileman *et al*., 2005), arabidopsis (Burch-Smith *et al*., 2006), columbine (*Aquilegia*; Gould and Kramer 2007), and bilberry (*Vaccinium myrtillus*; Jaakola *et al*., 2010).

Compared with other VIGS vectors, TRV based vectors have many advantages. TRV has a very wide host range and genes in TRV RNA2 are expressed individually from sgRNAs. The incorporation of heterologous insert sequences does not compromise the overall virus replication (MacFarlane, 2010). TRV induces strong and uniform gene silencing systemically through-out experimental plants, but the TRV vector itself does not induce severe symptoms that complicate the VIGS phenotypes (Ratcliff *et al*., 2001). How TRV can induce more intensive VIGS than other vectors is still not clear. It is likely that TRV 16K, which is a relatively weak silencing suppressor, balances between host silencing and virus silencing suppression well. This should allow for a good spread of both infection and silencing (MacFarlane, 2010).

Currently, there are three versions of TRV VIGS vectors, which are developed by the groups of Baulcombe (TRV-B; Ratcliff *et al*., 2001), Dinesh-Kumar (TRV-DK; Liu *et al*., 2002), and Lacomme (TRV-L; Valentine *et al*., 2004). In TRV-B and TRV-DK, gene 2b and 2c in TRV RNA2 were replaced by a multiple cloning site (MCS). Both vectors induced intensive gene silencing phenotypes in *N. benthamiana* and related species. TRV-DK seems to be effective on a broader host range and causes more rapid silencing. This is probably due to that it contains a subgenomic promoter upstream of the nonviral insert (Lu *et al*., 2003). The availability of the Gateway version of TRV-DK has made it more popular. Vector TRV-L retained the 2b gene in TRV RNA2, and induced stronger gene silencing in *Arabidopsis* root (Valentine *et al*., 2004).

1.5 Flavonoid biosynthesis

Flavonoids are plant-specific secondary metabolites that may accumulate in almost all tissues of plants. All flavonoids contain a C_6-C_3-C_6 carbon framework (Fig. 6A), and are synthesized through a branch of the general phenylpropanoid biosynthetic pathway that also produces lignins (Marais *et al*., 2008). The flavonoid biosynthetic pathway itself is also branched, and produces both colored pigments and colorless compounds (Fig. 6C). Depending on the modification of the B and C rings, flavonoids are classified into many subgroups, such as the chalcones, flavones, flavonols, flavandiols, anthocyanins, and pro-anthocyanins (Winkel-Shirley, 2001). Some plant species also produce some specialized forms of flavonoids, such as isoflavonoids (Fig. 6B,C) in legumes (Fabaceae), and phlobaphenes (Fig 6C) in maize (*Zea mays*) and sorghum (*Sorghum bicolor*).

The most well-known physiological functions of flavonoid products are as pigments (anthocyanins) and copigments (flavones and flavonols) to color flowers, fruits, seeds and leaves. They also play important roles in

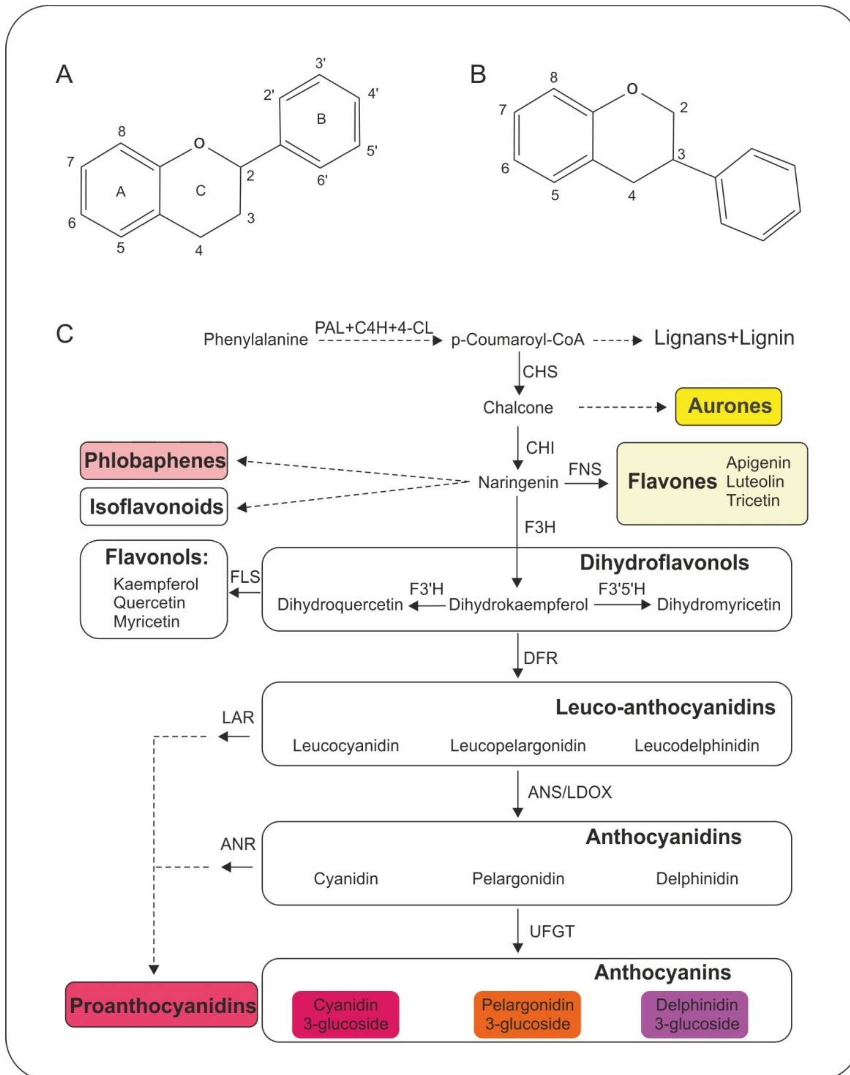

Fig. 6. Flavonoids and their biosynthesis. A and B, The C_6-C_3-C_6 carbon framework of flavonoids and isoflavonoids, respectively. C, The simplified flavonoid biosynthetic pathway. Products of the anthocyanin branch and the end products of other flavonoid subgroups are framed. Colored flavonoids, such as anthocyanins, proanthocyanidins, phlobaphenes, aurones were marked with their corresponding colors. Flavones (Apigenin, Luteolin and Tricetin) function as co-pigments, and are marked with a pale yellow. Enzyme names are abbreviated as follows: PAL, phenylalanine ammonia lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4 coumarate CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; *F3′H*, flavanone 3′-hydroxylase; *F3′5′H*, flavanone 3′5′-hydroxylase; DFR, dihydroflavonol reductase; FLS, flavonol synthase; ANS/LDOX, anthocyanidin synthase/leuco-anthocyanidin dioxygenase; UFGT, UDP-flavonoid glucosyl transferase; ANR, anthocyanidin reductase; LAR, leuco anthocyanidin reductase.

plant resistance against phytopathogens and herbivores, in signaling during nodulation, in male fertility of some plant species, and in auxin transport (Mol *et al*., 1998; Winkel-Shirley, 2002). From a human point-of-view, flavonoids also supply crucial and healthy ingredients for fruits, wine and chocolate. Due to their high antioxidant capacity, flavonoids are believed to have positive effects on blood vessels and cancer resistance (Kähkonen *et al*., 2003; Vinson *et al*., 2005; Dragsted *et al*., 2006; Butelli *et al*., 2008).

Our current understanding of the flavonoid biosynthetic pathway has mostly been obtained from studies on four models in the system: maize (*Zea mays*), snapdragon (*Antirrhinum majus*), petunia (*Petunia hybrida*), and arabidopsis (*Arabidopsis thaliana*). Through studying mutants that affect flavonoid biosynthesis, a number of structural and regulatory genes have been characterized, and the flavonoid biosynthetic pathway is well established (Holton *et al*., 1993; Mol *et al*., 1998). Starting from the substrate 4-Coumaroyl-CoA, chalcone synthase (CHS) functions at the entry point of the pathway. Subsequently, chalcone isomerase (CHI) catalyzes the isomerization of the chalcone to naringenin, from which all other classes of flavonoids are synthesized. The action of flavone synthases (FNS) and flavonol synthases (FLS) leads to the production of flavones and flavonols, respectively (Davies *et al*., 2003; Martens & Mithöfer, 2005). Reactions catalyzed by flavanone 3-hydroxylases (F3H), flavonoid hydroxylases (F3'H or F3'5'H), dihydroflavonol reductases (DFR), anthocyanidin synthases (ANS) and glycosyl transferases (GT) yield to colored anthocyanin pigments (Reviewed by Dooner & Robbins, 1991; Holton & Cornish, 1995) (Fig. 5C).

Many factors, such as temperature, light, nutrient status, wounding, water stress, and pathogen infection, can affect flavonoid biosynthesis (Christie *et al*., 1994; Dixon & Paiva, 1995; Chalker-Scott, 1999; Carbone *et al*., 2009). Mostly, the regulation of the flavonoid synthesis occurs via the coordinated transcriptional control of the structural genes. The combination of the three major transcription factors (TF) of R2R3-MYB, helix-loop-helix (bHLH) domains and a WD40 protein, and their interactions, determine the activation, spatial and temporal expression of structural genes, which in turn, regulate the biosynthesis of different classes of flavonoids and their distributions (Koes *et al*., 2005). Recently, some other proteins, such as TFs that contain MADS box, Zn-finger, and WRKY domains have also been reported that can regulate the flavonoid biosynthesis (Nesi *et al*., 2002; Johnson *et al*., 2002; Sagasser *et al*., 2002; Jaakola *et al*., 2010).

1.5.1 Chalcone synthase

CHS belongs to the type III polyketide synthase (PKS) superfamily, which also includes stilbene synthase (STS), 2-pyrone synthase (2PS), bibenzyl synthase (BBS), acridone synthase (ACS), and coumaroyl triacetic acid synthase (CTAS) (Flores-Sanchez & Verpoorte, 2008). Unlike type I and type II PKS that are found in bacteria and fungi, type III PKS is almost completely restricted to plants (Austin & Noel, 2003; Austin *et al*., 2004; Seshime *et al*., 2005). The type III PKS utilizes a catalytic mechanism that closely parallels fatty acid biosynthesis, but without the involvement of acyl carrier proteins (Abe & Morita, 2010). Type III PKSs of plant origin share a 46-95% similarity in their amino acid sequence identity (Austin & Noel, 2003; Abe *et al*., 2005). They have a common three-dimensional overall fold, and contain a conserved Cys-His-Asn catalytic triad in the internal active site (Abe & Morita, 2010). Only small modifications of few amino acids may significantly alter the binding pocket volume and redirect the enzyme's function (Ferrer *et al*., 1999; Jez *et al*., 2000).

CHS is one of the best studied plant-specific type III PKSs. This enzyme catalyses the stepwise condensation of one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA into naringenin chalcone, an important intermediate for the flavonoid biosynthesis. CHS differs from other plant specific type III PKSs in the: 1) selection of the start substrate; 2) the number of malonyl-CoA condensed; and 3) the mechanism of the cyclization reaction (Austin & Noel, 2003; Abe & Morita, 2010). The 3-dimensional structure has been well characterized for the alfalfa (*Medicago sativa*) CHS2 (Ferrer *et al*. 1999). In arabidopsis and snapdragon, CHS is encoded by a single gene (Sommer & Saedler, 1986; Burbulis *et al*., 1996). More commonly, CHS is encoded by a small multigene family, such as those in petunia (8-10 members) (Koes *et al*., 1989), maize (2 members) (Coe *et al*., 1981), morning glory (6 members) (Johzuka-Hisatomi *et al*., 1999), soybean (9 members) (Tuteja & Vodkin, 2008), and dahlia (2 members) (Ohno *et al*., 2011). Guarding the entry point of the flavonoid biosynthetic pathway, loss of CHS enzyme activities results in albino flowers or fruits that lack all flavonoid pigments (Napoli *et al*., 1990; Schijlen *et al*., 2007; Ohno *et al*., 2011; Morita *et al*., 2012; Dare *et al*., 2013).

Like other structural genes in the flavonoid biosynthetic pathway, CHS expression is regulated spatially and temporally by developmental, environmental and stress stimuli. In most species, CHS is expressed specifically in flowers and fruits where the anthocyanin pigments concentrate, and is under developmental control in those tissues (Koes *et al*., 1989; Jackson *et al*., 1992; Zhou *et al*., 2011). In other non-pigmented tissues, such as leaves and stems, *CHS* can be induced by environmental stress factors (Dixon *et al*., 1986; Dao *et al*., 2011). Individual members of

the *CHS* multigene family can be differentially regulated, and show different tissue- and development-specific expression patterns (Dangle *et al*., 1989; Tuteja *et al*., 2004; Yi *et al*., 2010). In some species, such as legumes, flavonoids play a key role in the activation of the nodulation process. Thus, CHSs in those species are also highly expressed in roots (Tuteja *et al*., 2004; Yi *et al*., 2010).

1.5.2 Flavonoid biosynthesis and chalcone synthase in Gerbera hybrida

A wide range of flower and inflorescence colors is an important trait that makes gerbera one of the most popular ornamental plants. From the collections of a single breeder (Terra Nigra B.V.), one can find more than 100 gerbera cultivars with flowers in different color patterns, such as white, yellow, red, pink, purple, and brown (www.terranigra.com/). Gerbera flower pigmentation is based on the interaction of carotenoids and flavonoids (Tyrach, 1994). Cultivars with carotenoids are yellow, whereas acyanic cultivars contain neither carotenoids nor anthocyanins. The major flavonoids in pigmented cultivars are pelargonidin and cyanidin (anthocyanins), apigenin and luteolin (flavones), and kaempferol and quercetin (flavonols) (Tyrach & Horn, 1997).

The flavonoid biosynthetic pathway in gerbera has not yet been fully elucidated. However, based on our current understanding, the flavonoid biosynthetic pathway in gerbera follows previously proposed models well (Donner & Robbins, 1991; Koes *et al*., 2005). By the screening of a gerbera flower cDNA library, genes that encode gerbera CHSs and DFRs were isolated early (Helariutta *et al*., 1993, 1995a,b). GCHS1 is a typical CHS that catalyzes the reaction that converts 4-coumaroyl-CoA and malonyl-CoA substrates into naringenin chalcone (Helariutta *et al*., 1995b). The expression of both *GCHS1* and *GDFR* are epidermal specific in flower petals, and correlate with the anthocyanin accumulation during the petal development (Helariutta *et al*., 1993, 1995b). After knocking down *GCHS1*, anthocyanin accumulation was inhibited in the stable anti-sense transgenic lines (Elomaa *et al*., 1993). GCHS3 is also a true CHS, but has a distinct expression pattern. Spatially, GCHS3 expression is mostly concentrated in the pappus bristles, with small amounts in earlier stages of the petals (Helariutta *et al*., 1995b).

GCHS2 was first described as a CHS-like gene, which shares 73% deduced amino acid sequence identity with GCHS1 and GCHS3, and about 70% with alfalfa CHS2 and arabidopsis CHS. However, the expression pattern of *GCHS2* is unexpectedly broad as it occurs almost in all tissues of gerbera (Helariutta *et al*., 1995b). In the enzyme activity assay, GCHS2 did not use 4-coumaroyl-CoA as a start substrate, but it did

recognize acetyl-CoA which led to the production of triacetolactone (TAL). TAL is the candidate precursor for both gerberin and parasorboside, two bitter glucosidic lactones that are found in all gerbera tissues (Helariutta *et al*., 1995b; Eckermann *et al*., 1998). Subsequently, GCHS2 was renamed as G2PS1 (Eckermann *et al*., 1998). Through the comparison the 3-dimensional structures, Ferrer *et al*. (1999) revealed that G2PS1 has a much smaller substrate-binding pocket (269 $Å^3$) than alfalfa CHS2 (923 $Å^3$), which explains why G2PS1 uses a smaller molecular than 4-coumaroyl-CoA as a starter substrate.

Some other enzymes in the flavonoid pathway were also isolated in gerbera, mostly by Martens and his colleagues. Gerbera FNS II, function in the branched pathway to synthesize of flavone, was the first functional FNS II that was isolated from plant species (Martens & Forkmann, 1999). Besides, genes that encode an *ANS* (Wellmann *et al*., 2006), a *F3H* (Martens, unpublished), and a *F3'H* (Seitz *et al*., 2006) were also isolated, and their chemical functions were identified. In addition, two genes that encode regulatory proteins have been isolated. *GMYC1* encodes a bHLH type regulator. Together with the petunia MYB partner *AN2* (Quattrocchio *et al*., 1999), *GMYC1* was found to activate the gerbera *DFR* promoter in a transient assay (Elomaa *et al*., 1998). *GMYB10* encodes a R2R3-MYB regulator, which can activate the anthocyanin biosynthesis in transgenic tobacco (Elomaa *et al*., 2003). The overexpression of *GMYB10* in transgenic gerbera plants significantly enhanced pigmentation accumulation, and induced cyanidin biosynthesis in the cultivar Terraregina, which is normally characterized by pelargonidin containing flowers (Laitinen *et al*., 2008).

2. AIMS OF THE STUDY

The aim of this study was to develop a feasible VIGS system for functional studies in gerbera. TRV is naturally a gerbera pathogen (Stouffer, 1965) and an excellent VIGS vector. It was selected as the vector to be developed. Preliminary tests, by the infection of gerbera cultivar Terraregina with two previously developed TRV vectors (Ratcliff *et al*., 2001; Liu *et al*., 2002), did not result in efficient *PDS* silencing. TRV RNA2, as a carrier of host gene fragments for target gene silencing, could not spread systemically with ease. Thus, the aim of this study also included developing new VIGS vectors based on TRV RNA1.

Specifically, we aimed at achieving the following objectives:

1) to study the functions of TRV 16K on virus infection, and to develop new TRV vectors for gerbera by partial deletions of TRV 16K gene.

2) to develop an efficient VIGS system for gerbera based on TRV VIGS vectors.

3) to characterize functions of gerbera CHS gene family members using VIGS.

3. MATERIALS AND METHODS

Materials and methods used in this study are summarized in Table 1. All details have been described in the original publications (I, II, III).

Table 1 Materials and methods that have been used in this study. The roman numerals refer to the three original publications.

Materials or methods	Publication
Nicotiana benthamiana plants	I
Gerbera plants	II, III
TRV constructs	I, II, III
Construction of TRV vectors	I, II, III
Virus infection and agroinfiltration	I, II, III
Northern blotting	I
qRT-PCR	I, II, III
GFP imaging	I
Scanning electron microscopy (SEM)	II
Norflurazon treatment*	II
In situ hybridization	III
CHS enzyme assay	III
Isolation of polysome RNA	III
Western blotting	III
HPLC analysis of flavonoids*	III

Note: Methods marked with a star were conducted by the co-authors.

4. RESULTS AND DISCUSSION

4.1 TRV 16K helps in balancing the induction and suppression of the host anti-viral RNA silencing (I)

TRV, as an excellent VIGS vector, is well known for its inducing uniform and intensive target gene silencing phenotypes in many plant species (MacFarlane, 2010). TRV induced disease symptoms are mild compared with most of the other viruses (Ratcliff *et al*., 2001). This is an important characteristic for a VIGS vector because viruses themselves may induce symptoms that disturb the interpretation of VIGS phenotypes. TRV 16K, a weak RNA silencing suppressor (Ghazala *et al*. 2008; Martín-Hernández and Baulcombe 2008; Martínez-Priego *et al*. 2008), plays an essential role in balancing virus invasion, host anti-viral silencing, and suppression of the silencing.

4.1.1 Disruption of 16K enhanced viral symptoms

To study the function of 16K on TRV infection and TRV VIGS, we generated three TRV RNA1 constructs, in which the *16K* expression was disrupted. 16Kstop carries a premature stop codon in the beginning of *16K* ORF. M1 and M2 contain partial deletions in the *16K* ORF, but still include the premature stop codon (Fig. 1 in paper I). Infection experiments showed that all three constructs were capable of infecting *N. benthamiana* systemically (Fig. 3 in paper I). The disruption of 16K did not affect virus replication in the infiltrated leaves (Fig. S3 in paper I), but it did retard the virus long-distance movement (Fig. 3 in paper I). This is consistent with a recent study (Martín-Hernández and Baulcombe 2008), but conflicts with the findings of two other studies (GuilFord *et al*., 1991; Liu *et al*., 2002). In one study, the authors suggested that 16K protein was not essential for any process of TRV NM-type infection (Guilford *et al*., 1991), and in the other study, the authors showed that the disruption of 16K slowed down the virus replication, and almost totally eliminated virus systemic movement (Liu *et al*., 2002). We are not sure about the reasons for the discrepancies in the behaviors of 16K mutants. They may be caused by different conditions for plant growth, or different TRV clones where the 16K mutants originated.

Unexpectedly, the TRV 16K mutants, without the expression of the 16K RNA silencing suppressor, induced more severe necrosis in the infiltrated leaves and also the general disease symptoms in infected plants (Fig. 7 and Fig. 2 in paper I). Although associated with a certain reduction in systemic spread, the 16Kstop construct was almost as vigorous as the

wtRNA1, and systemically infected all infiltrated plants. M1 and M2 were relatively weak, and caused systemic infection in about one third of the infiltrated *N. benthamiana* plants. However, once plants became systemically infected by the 16K mutants (16Kstop, M1 or M2), they all developed severe necrosis symptoms. Those infected by 16Kstop developed the most severe necrosis, in almost all leaves (Fig. 7).

Thus, 16K is essential for TRV to do efficient infection, and to avoid inducing destructive symptoms.

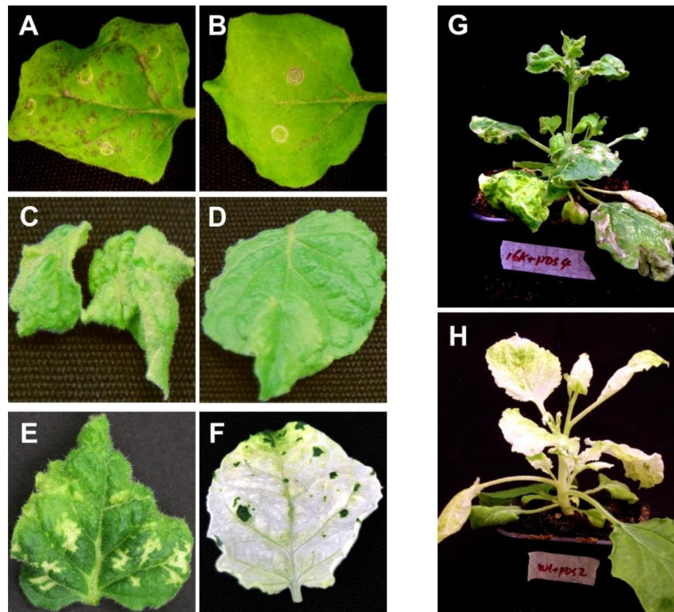

Fig. 7. Disruption of 16K enhanced TRV induced symptoms in *Nicotiana benthamiana*, but reduced TRV-induced silencing. A and B, Infiltrated leaf inoculated with 16Kstop+RNA2 (A), or wtRNA1+RNA2 (B). C and D, Leaves were systemically infected with 16Kstop+RNA2 (C), or wtRNA1+RNA2 (D). E and F, *Phytoene desaturase* (*PDS*) silencing induced by 16Kstop+RNA2:PDS (E), or wtRNA1+RNA2:PDS (F). G and H, the overall symptoms induced by 16Kstop+RNA2:PDS (G), or wtRNA1+RNA2:PDS (H).

4.1.2 16K enhances PDS silencing

As planned, mutants M1 and M2 were tested for VIGS in *N. benthamiana*. Two constructs, M1:PDS and M2:PDS were developed to carry a 200 bp of *N. benthamiana PDS* sequence. Combinations of M1:PDS+RNA2 and M2:PDS+RNA2 induced a similar and mild photobleaching phenotype in about one third of the infected *N. benthamiana* plants. The *PDS* mRNA in

the photobleached area decreased to 65% of the level detected in the control sample. Even so, the original TRV VIGS system (Liu *et al*., 2002), with the combination of wtRNA1+RNA2:PDS, induced extensive *PDS* silencing, with the *PDS* mRNA in the photobleached area having decreased to 4% of the level of the control (Fig. 4A in paper I).

TRV 16K mutants, combined with RNA2:PDS, were also tested for their VIGS efficiency. M1+RNA2:PDS and M2+RNA2:PDS induced similar weak *PDS* silencing, which was observed in most of the infected plants. 16Kstop+RNA2:PDS induced relatively more intensive *PDS* silencing in all infiltrated plants. However, none of the combination induced as uniform and intensive *PDS* silencing as wtRNA1+RNA2 (Fig. 4B in paper I).

4.1.3 16K stabilizes recombinant RNA2 genome

In order to monitor timely TRV movement, RNA2:GFP was constructed so that *GFP* was expressed and translated as a viral protein (Fig. 1 in paper I; MacFarlane & Popovich, 2000). *N. benthamiana* plants infected with wtRNA1+RNA2:GFP showed intensive GFP fluorescence in the systemically infected leaves 6 days post infiltration (dpi). In contrast, plants infected with M1+RNA2:GFP and 16Kstop+RNA2:GFP did not show any GFP inflorescence until 16 dpi, although apparent viral symptoms could be observed as early as 6 dpi (Fig. 5 in paper I). RNA blotting detected only truncated forms of the RNA2:GFP genome in leaves systemically infected with M1+RNA2:GFP and 16Kstop+RNA2:GFP. RT-PCR with primer pair flanking the *GFP* insert and subsequently sequencing of the PCR products showed that partial or whole of the introduced heterologous *GFP* insert was lost from the RNA2:GFP progeny viruses in the infection of M1+RNA2:GFP and 16Kstop+RNA2:GFP. In addition, viral sequences neighboring the *GFP* insert were sometimes lost (Fig. 5 in paper I). In contrast, progeny viruses of RNA2:GFP in plants infected with wtRNA1+RNA2:GFP were mostly detected in full size (Fig. 5 in paper I).

In general, 16K is essential for TRV to do efficient infection, to stabilize the recombinant RNA2 genome, to achieve intensive VIGS, and to alleviate destructive viral symptoms. In addition, 16K also helps TRV virus to enter the apical meristems (Martín-Hernández and Baulcombe 2008). All those characteristics favor TRV as an excellent VIGS vector. Our study also suggested that it is not practical to develop new VIGS vectors based on TRV RNA1 by the disruption of 16K.

4.2 TRV 29K movement protein is an RNA silencing suppressor (I)

Suppressing the antiviral RNA silencing by virus encoded VSR is a general strategy for viruses to fulfill their infection. Accordingly, the lack or inactivation of VSRs always leads to recovery phenotypes in the infected plants. All viruses that have been closely screened so far contain VSRs (Li & Ding, 2006). Some viruses, such as CTV and geminiviruses, are known to encode multiple VSRs in their genomes (Lu *et al*., 2004; Vanitharani *et al*., 2004). Previous studies have demonstrated that TRV 29K was associated with TRV induced symptoms (Ziegler-Graff *et al*., 1991; Ghazala & Varrelmann, 2007). Results of this study showed that all three TRV 16K mutants were competent for systemic infection in *N. benthamiana*, and induced even more severe necrosis in infected plants. This prompted us to speculate that in addition to the 16K, TRV might encode a second VSR in the RNA1 genome.

To verify our hypothesis, a “silencing on the spot” agroinfiltration assay (Johansen & Carrington, 2001) was first conducted in the leaves of GFP-transgenic *N. benthamiana line* 16c (Brigneti *et al*., 1998). The cDNAs of TRV 29K, 16K, TMV 30K, HC-Pro of *Potato virus A* (PVA) were cloned into a pA binary vector (Savenkov & Valkonen, 2001), under the control of the CaMV 35S promoter. Leaves of GFP 16c plants were co-infiltrated with *Agrobacterium* carrying the GFP and pA constructs (pA:29K, pA:16K, pA:30K, and pA:HC-Pro). TRV 29K and TMV 30K did not suppress GFP silencing. In contrast, TRV 16K and PVA HC-Pro prevented GFP silencing and supported strong GFP expression (Fig. S4 in paper I).

Co-infiltration of *Agrobacterium* carrying TRV RNA1 infectious constructs and GFP expression construct, however, showed that TRV 29K has the ability to suppress GFP silencing. All three RNA1 16K mutants (M1, M2, and 16Kstop), without the expression of 16K protein, suppressed GFP silencing, though with a relatively lower efficiency than that of wtRNA1 (Fig. 7 in paper I). The TRV trv30K16Kstop double mutant, after disruption both 16K and 29K, was unable to suppress GFP silencing (Fig. 7 in paper I).

Thus, TRV 29K is a multifunctional protein: besides as a movement protein, it also acts as an RNA silencing suppressor. The RNA silencing suppression by 29K, however, is associated with the multiplication of RNA1. This may be the first finding that a VSR is dependent on virus replication. The feature of silencing suppression by 29K suggests again that false results may have been deduced from the popular co-infiltration assay, by which the targeted protein was expressed alone. The coat protein (CP) encoded by *Citrus tristeza virus* (CTV) is another example, which did not suppress GFP silencing in the co-infiltration assay, but

prevented intercellular GUS silencing in the grafting experiments (Lu *et al*., 2004).

These results showed that TRV is one of the few viruses that encode multiple RNA silencing suppressors. Other examples of viruses with multiple silencing suppressors are CTV with three VSRs (Lu *et al*., 2004), *Potato chlorotic stunt virus* (SPCSV) with two VSRs (Kreuze *et al*., 2005), and PVA with two VSRs (Brigneti *et al*., 1998; Rajamäki & Valkonen, 2010), each of which works by a distinct mode of action. TRV 29K and 16K also use different mechanisms in suppression silencing. Although 16K has the ability to suppress RNA silencing independently, 29K functions only in association with virus replication. In addition, our results also showed that 16K and 29K function cooperatively in alleviating host responses (necrosis) that may harm the virus.

4.3 Application of TRV VIGS in gerbera (I, II)

4.3.1 Factors affecting VIGS in gerbera

It is known that TRV is able of infecting *Gerbera jamesonii* (Stouffer, 1965). As a VIGS vector, TRV has many advantages over other VIGS vectors. Thus, we selected TRV for developing a VIGS vector for gerbera.

Our first VIGS experiment, however, did not succeed, when TRV-DK was used in the cultivar Terraregina. RT-PCR results showed that TRV RNA2, as a main carrier of host gene fragments for gene silencing induction, was not able to spread systemically in cultivar Terraregina, although TRV RNA1 did show systemic spreading. This prompted us to develop new TRV VIGS vectors, and to use TRV RNA1 as a carrier for host gene fragments. However, the two newly developed vectors M1 and M2 induced only mild gene silencing, due to the disruption of the 16K, a protein that turned out to be essential for efficient VIGS.

It has been shown that genetically different cultivars of the same species behaved differently in response to TRV infection (Ghazala & Varrelmann, 2007; MacFarlane, 2010) and TRV VIGS (Chen *et al*., 2004). We therefore screened 21 gerbera cultivars (Table S1 in paper II) for their responses to TRV VIGS (Fig. 1 in paper II). The cultivar Terraregina, that is routinely used in our laboratory for stable transformation and large-scale sequencing, showed almost no silencing phenotypes following the syringe infiltration of TRV:GPDS. Five other cultivars, however, showed photobleaching symptoms by silencing of the gerbera *PDS* (Fig. 1b in paper II). Among them, the cultivar Grizzly showed the most intensive *PDS* silencing symptoms, followed by the cultivars White Grizzly and

Lamborghini. The photobleaching phenotype became visible as early as 12 dpi in Grizzly, and 16-20 dpi in other cultivars. On the other hand, the cultivar President was most sensitive to TRV infection, with 12 out of 18 treated plants showed photobleaching at some level. Similar results were also observed in the study of paper II, where *GCHS1* silencing phenotypes in President inflorescences were achieved more frequently and in larger sectors than those of Terraregina inflorescences. Nevertheless, the cultivar President also showed the most severe virus symptoms in leaves, which made it unsuitable for VIGS in leaves. Both TRV RNA1 and RNA2:GPDS were detectable from the photobleached area in the systemically infected leaves. Thus, TRV RNA2 was able to move systemically in those five cultivars.

Optimization of other conditions also favoured TRV VIGS in gerbera. Vacuum infiltration is amenable for small seedlings, and induced more intensive PDS silencing than syringe infiltration. All cultivars subjected to vacuum infiltration showed strong *PDS* silencing phenotypes, even the cultivar Terraregina that was hardly infected by syringe infiltration (Fig. 1c in paper II). Vacuum induced *PDS* silencing persisted for more than one year in all cultivars, and also resulted photobleaching in newly developed scapes and inflorescence bracts (Fig. 1d,e in paper II). Vacuum infiltration has also been shown to have great efficiency at inducing gene silencing in other species, such as *Thalictrum*, *Jactropha curcas*, *Arabidopsis*, and *Papaver somniferum* (Hileman *et al*., 2005; Wang *et al*., 2006; Ye *et al*., 2009; Di Stilio *et al*., 2010). Nevertheless, vacuum treatment sometimes caused severe injury of gerbera seedlings in our study, which delayed plant growth and VIGS symptom development. Thus, the vacuum pressure should be controlled for high seedling survival rate and optimal silencing.

The developmental stage of the plant at the time of inoculation is critical for VIGS (Hileman *et al*., 2005; Burch-Smith *et al*., 2006). We divided gerbera vegetative development into 3 stages (Fig. 1a in paper II). Syringe infiltration gerbera seedlings at stage 1 induced the most intensive silencing in all gerbera cultivars. Remarkably, the cultivar Terraregina that showed no photobleaching phenotypes previously (syringe infiltration at stage 2), developed tiny photobleaching patches in 2 out of 12 inoculated seedlings when syringe infiltrated at stage 1.

For silencing genes on gerbera inflorescences, large sized mature gerbera plants need to be used, which makes the vacuum infiltration method impractical. To obtain desirable silencing in flowers, we inoculated *Agrobacterium* on young inflorescence buds (about 2 cm long) by wound scratching the surface of scapes. VIGS silencing phenotypes were normally developed in sectors on the same side of the inflorescence at the site where scratching inoculation was done.

Other attempts by increasing *Agrobacterium* concentration from OD_{600} 1.0 to 4.0 did not enhance *PDS* silencing in gerbera. Moreover, the "Agrodrench" method that is suitable for large-scale VIGS in Solanaceous species (Ryu *et al*., 2004), failed to induce gene silencing in gerbera.

4.3.2 TRV VIGS in gerbera

Using the selected gerbera cultivars and the optimized conditions, we succeeded in silencing three genes (*GPDS*, *GCHL-H*, and *GCHL-I*) in vegetative tissues, and two genes (*GCHS1* and *GGLO1*) in gerbera inflorescences. Gerbera PDS and Mg-chelatase are important enzymes that are involved in carotenoid and chlorophyll biosynthesis respectively (Kumagai *et al*., 1995; Walker & Willows, 1997). Gerbera CHS1 is a key enzyme in the anthocyanin biosynthesis pathway (Helariutta *et al*., 1995b). After silencing of those 4 genes, gerbera plants developed clear visible phenotypes (colour changes). Thus, they all turned out to be useful markers for VIGS in gerbera. Gerbera *GLO1* is a B function MADS-box gene, which is involved in petal and stamen development (Yu *et al*., 1999; Broholm *et al*., 2010). Silencing of *GGLO1*, gerbera developed inflorescences similar to those in stable *GGLO1*-antisense transgenic lines (Yu *et al*., 1999).

Marker genes are useful for tracing VIGS development, especially when silencing of a target gene does not give any visible phenotypes (Quadrana *et al*., 2011). However, suitable marker genes for VIGS should be carefully selected to avoid any possible impact on target gene expression by the silencing of the marker genes. We showed in this study that *G2PS1* expression was down regulated by the silencing of gerbera *PDS* and *Mg-chelatase* subunits. Nevertheless, none of the markers seem to have obvious connections with the G2PS1 catalysed gerberin and parasorboside biosynthesis pathway (Eckermann *et al*., 1998).

4.4 Functional characterization for gerbera CHSs (III)

4.4.1 Gerbera CHS is represented by a three-gene family

CHS enzyme belongs to type III PKS super family, and is the first committed enzyme in the flavonoid biosynthesis pathway that catalyzes the stepwise synthesis of chalcone. The function of CHS determines the substrate flow between the flavonoid pathway and other competing branches of the general phenylpropanoid pathway. This leads to a diversity of phenolic compounds, such as lignins and anthocyanins (Winkel-Shirley

2001; Vogt, 2009). Plant CHSs share high similarity in amino acid sequence identity, and also a common 3-dimensional structure (Abe & Morita, 2010). CHS in arabidopsis, parsley, and snapdragon is encoded by a single gene, though CHS is encoded by a multi-gene family in most of the angiosperms (Dao *et al*., 2011).

In this study, we characterized in gerbera the three-membered *CHS* gene family, including the *GCHS4* that was not studied in detail earlier. Analysis of amino acid sequences (Fig. 1 in paper III) showed that all sequences of *GCHS4*, *GCHS1*, *GCHS3*, alfalfa *CHS2*, and *G2PS1* comprise the 4 residues (Cys 164, Phe 215, His 303, and Asn 336; all residues numbers as in alfalfa CHS2) that are shown to be absolutely conserved in all type III PKSs, and the other 13 residues (Pro 138, Gly 163, Gly167, Leu 214, Asp 217, Gly 262, Pro 304, Gly 305, Gly 306, Gly 335, Gly 374, Pro 375, and Gly376) that shape the geometry of the active site (Ferrer *et al*., 1999). In addition, GCHS4 and the other three CHSs also contain the 3 residues that are essential for the coumaroyl-binding pocket (Thr 197 and Ser 338) and the cyclization pocket (Gly 256). Those three residues in *G2PS1* are substituted by Leu, Leu, and Ile, respectively.

Enzyme activity assays further confirmed that GCHS4 is a true CHS similar to GCHS1 and GCHS3. They all converted 4-coumaroyl-CoA and malonyl-CoA substrates into naringenin chalcone (Fig. 2 in paper III).

4.4.2 Spatial and temporal expression patterns of gerbera CHSs

Expression of gerbera *CHSs* is regulated both spatially and temporally. In the 11 screened tissue types of cultivar Terraregina, gerbera *CHSs* were mostly expressed in the reproductive tissues, where flavonoids accumulate (Fig. 3 in paper III). *GCHS3* was predominantly expressed in pappus, whereas *GCHS1* in pappus and petals, and *GCHS4* in petals and carpels. The *CHS*-like gene, *G2PS1*, had the broadest expression pattern, and was highly expressed in almost all tissue types. The CHSs expressed in petals are supposed to be involved in pelargonidin and cyanidin biosynthesis. In the cultivar Terraregina, due to the loss of DFR expression (Helariutta *et al*., 1995a), CHSs expressed in pappus are involved in the biosynthesis of colorless flavonoids. Unlike *CHSs* in soybean that are abundantly expressed in root tissues (Tuteja *et al*., 1994), gerbera *CHS* transcript was not detected in roots in our study.

In addition to flower organs, gerbera basal parts of leaf petioles and scape are naturally red and contain cyanidin (Fig. 4 in paper III). *GCHS4* was the only *CHS* that was expressed in the red part of those vegetative tissues, and thus was inferred to be responsible for cyanidin biosynthesis in those sites. Under stress conditions, gerbera leaf blade becomes

reddish and is rich in cyanidin. Again, *GCHS4* is the only *CHS* that is induced by the stress stimuli in leaves and it mediates cyanidin biosynthesis there. GMYB10, an important TF that regulates gerbera *CHS* expression (Laitinen *et al*., 2008), was highly up regulated in the stressed leaves, but was expressed at similar levels in both red and green tissues of leaf petioles and inflorescence scapes (Fig. 4 in paper III). The induction of GCHS4 in petioles and scapes, however, also relies on the regulation of *GMYB10*, because in *GMYB10* antisense transgenic gerbera lines, both petioles and scapes were green and lacked any anthocyanin pigments (Fig. S1 in paper III). Thus, GCHS4 is the dominant CHS in gerbera leaves and scapes, which is inducible under environmental cues and is under the regulation of *GMYB10* transcription factor.

Gerbera *CHS*s in petals are developmentally regulated. Each of them has a distinct expression pattern. Consistent with the spatial expression patterns, expression of *GCHS3* was negligibly low in all 11 developmental stages of petals. *G2PS1*, once more, was highly expressed in all stages, with the peak levels at stage 6-9. *GCHS1* showed a typical *CHS* expression pattern that is similar to these CHS patterns in petunia, snapdragon, and tree peony (*Paeonia suffruticosa*). In these species, the expression levels of CHS first increasing in early stages, peaking at middle stages when flowers pigmentation starts, and decreasing gradually at later stages (Koes *et al*., 1989; Jackson *et al*., 1992; Zhou *et al*., 2011). The expression pattern of *GCHS4* is also temporally correlated with petal pigmentation, but its expression started later at stage 4, peaked at stage 6, and kept at high levels in all later stages.

4.4.3 Silencing **GCHS1** *and* **GCHS4** *separately by VIGS revealed that* **GCHS1** *is the major* **CHS** *in gerbera petals for anthocyanin biosynthesis*

Although most plant species contain multi copies of *CHS*s in their genome, normally only one or two *CHS* members are expressed, or mRNAs of one or two members account for the majority of the total *CHS* transcripts (Koes *et al*., 1986; Fukada-Tanaka *et al*., 1997; Johzuka-Hisatomi *et al*., 1999). This is also the case for gerbera. In gerbera petals, two of the three CHS genes, the *GCHS1* and the *GCHS4*, *are* expressed. The expression patterns of those two genes are temporally correlated with anthocyanin accumulation. The peak expression level of *GCHS4* is about twice as high as that of *GCHS1* (Fig. 3 in paper III). To ascertain which of them is responsible for anthocyanin biosynthesis in gerbera petals, *GCHS1* and *GCHS4* were silenced separately by TRV VIGS. By using VIGS vectors carrying gene specific fragments, gene specific silencing was achieved in most of the screened inflorescences (Fig. 5 in paper III).

In the cultivar Terraregina, the knock down of *GCHS1* led to the development of albino petals (Fig. 5a in paper III). All major flavonoid products, such as pelargonidin, apigenin, and kaempferol were significantly decreased compared with those in the control samples (Table 3 in paper III). In contrast, silencing of *GCHS4* did not induce any visible phenotypes in Terraregina petals, and the major flavonoid compounds were at similar levels to those in the control samples.

The Terraregina is a pelargonidin cultivar, which accumulates primarily pelargonidin anthocyanins in its petals. To check whether GCHS1 is specifically involved in pelargonidin biosynthesis and GCHS4 in cyanidin biosynthesis, the same VIGS experiment, to silence *GCHS1* and *GCHS4* independently, were done on the cyanidin containing cultivar President. Similar results were achieved as those in the cultivar Terraregina. After the silencing of *GCHS1*, flower developed white petals, with all major flavonoids significantly decreased. After silencing of *GCHS4*, no significant changes were observed on flower color or the levels of flavonoid compounds.

Despite *GCHS4* mRNAs taking up the majority *CHS* transcripts in gerbera petals, GCHS1 is the predominant functional CHS at that site, and is involved both in pelargonidin and cyanidin biosynthesis.

4.4.4 GCHS4 is regulated post-transcriptionally in gerbera petals

Structural genes in the flavonoid biosynthetic pathway are mostly regulated transcriptionally, by the combination and interaction of the R2R3-MYB, bHLH and WD40 TFs (Koes *et al*., 2005). Regulation in the post-transcriptional level has been reported only in a few cases (Pollak *et al*., 1995; Pairoba & walbot, 2003). In this research, we provided evidence that the *GCHS4* is regulated post-transcriptionally in gerbera petals.

GCHS4 is predominantly transcribed in gerbera petals, especially in the later stages 5-11. However, the silencing of *GCHS4* by VIGS had no significant effect on flower color or major flavonoid productions in the cultivars Terraregina and President. In addition, the enzyme activity in the petal stages (stage 1 to 11) follows well the *GCHS1* expression pattern but not the *GCHS4* expression pattern, indicating that *GCHS1* enzyme takes up the major share of the total CHS activity. The polysome experiments, however, showed that both *GCHS1* and *GCHS4* mRNAs were loaded on polysomes for translation. Thus, the regulation of *GCHS4* occurs after mRNA loading (to polysome), by the inhibition of translation, or through protein degradation.

5. CONCLUSIONS AND PROSPECTS

The results presented in this study demonstrated that the 16K encoded by TRV RNA1, as a cysteine-rich VSR, was required for TRV long distance spreading and efficient VIGS, and helped in maintaining the integrity of RNA2:GFP. In addition, the 29K MP manifested its ability to suppress RNA silencing. Unlike the 16K that has ability to suppress RNA silencing alone, 29K VSR did not. The RNA silencing suppressor ability of 29K is associated with replication of the RNA1.

The mechanism of 16K and 29K in suppressing RNA silencing, still, remains to be determined. Previous studies have shown that TRV 16K was able to block the local RNA silencing induced by single- and double-stranded RNA (dsRNA) (Martín-Hernández *et al*., 2008), indicating that 16K interfered with a step downstream of dsRNA formation in the RNA silencing pathway. On the other hand, the observation that 16K affected the accumulation of GFP-specific siRNAs, suggested that 16K targeted to a step upstream of siRNA production (Ghazala *et al*., 2008). Thus the 16K probably targets at the point of dsRNA procession (into siRNAs), by inhibiting host DCL activity directly, or by interactions with proteins that required for functioning of DCLs. Results of this study revealed that the 29K also blocked the accumulation of GFP specific siRNAs, which indicates that the 29K may target components upstream of the siRNA production.

We also demonstrated the efficient use of TRV VIGS for functional studies in gerbera. Intensive silencing was achieved by the silencing of *GPDS*, *GCHL-I*, and *GCHL-H* in green tissues, and *GGLO1* in inflorescences. Remarkably, *GCHS1* and *GCHS4* that share high sequence similarity were knocked down separately by TRV VIGS. GCHS1 turned out to be the major CHS in gerbera petals that functions for pigment biosynthesis, although both *GCHS1* and *GCHS4* are considerably expressed.

Gerbera belongs to Asteraceae, one of the largest families among flowering plants with about 22 000 species. Expanding the use of VIGS to gerbera is valuable for functional genomics research on species of the whole family, and will further facilitate comparative studies to better understand the evolution of developmental and metabolic diversity.

Gerbera plants are normally vigorous, grow fast, and possess large leaves and strong root systems when they are mature. Although TRV VIGS is able to silencing genes both in gerbera vegetative and inflorescence tissues, TRV is still relatively weak and often cannot easily achieve systemically movement throughout the infected plants. VIGS induced symptoms were thus often patchy or limited only within the infiltrated

inflorescences. In the future, other stronger VIGS vectors, such as *Apple latent spherical virus* (ALSV), *Alternanthera mosaic virus* (AltMV), and *Potato virus A* (PVA), could be tested for VIGS in gerbera. Alternatively, a helper construct that expresses a VSR could be co-infiltrated together with the TRV VIGS vector, in order to assist TRV to initiate its infection.

Questions have been raised when the *G2PS1* was knocked down by the silencing of *GPDS* and *Mg-chelatase*. PDS is involved in carotenoid synthesis, and Mg-chelatase in chlorophyll synthesis. Neither of them seems to have an apparent connection with the 2PS catalysed gerberin and parasorboside biosynthetic pathway. In one of our other studies, the overexpression of *G2PS1* in *Nicotiana tabacum* cv. SR1 resulted in the development of photobleached leaves, which was similar as those induced by the silencing of *GPDS* and *Mg-chelatase* in gerbera (Teemu Teeri and Paula Elomaa, unpublished results). The possible connections between *G2PS1* involved pathway, and *GPDS* or *Mg-chelatase* involved pathway need to be further investigated.

This study has revealed that the *GCHS4* in gerbera petals is post-transcriptionally regulated. This is one of a few cases that show post-transcriptional regulation of anthocyanin related genes in plants. Apparently, functional proteins of GCHS4 are available in gerbera vegetative tissues for cyanidin biosynthesis. In contrast, in gerbera petals, active GCHS4 enzymes were rarely detected, although *GCHS4* was actively transcribed and its mRNAs were comprehensively loaded onto polysomes for translation. The exact mechanism of post-transcriptional regulation of *GCHS4* in gerbera petals remains to be addressed.

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