MOLECULAR ANALYSIS OF POST-TRANSCRIPTIONAL GENE SILENCING: MECHANISMS AND ROLES

Molecular analysis of post-transcriptional gene silencing: mechanisms and roles

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ABSTRACT

This work is an investigation of post-transcriptional gene silencing (PTGS) in plants, a process that mediates sequence-specific degradation of RNA. Initially discovered in transgenic plants, PTGS has been long regarded as a curiosity, or even as an artefact of transgenesis. It is shown here that virus-induced gene silencing, in which recombinant viruses carrying element of the host genome trigger PTGS of the corresponding plant gene (Chapter one), is a manifestation of a defence system. This defence is remarkable in its ability to adapt to potentially any virus because its specificity is not genetically programmed by the host but, instead, is dictated by the genome sequence of the viral intruder itself. It is demonstrated in chapters 4 and 5 that PTGS of a transgene can spread in plants from one part to another, indicating the existence of a systemic, sequence-specific silencing signal that is likely to have a nucleic acid component. From the demonstration that replication of potato-virus X also triggers production of a silencing signal in non-transgenic plants (Chapter 8), it is proposed that this long-distance signalling process represents the systemic arm of the host PTGS defence response. Collectively, these findings define the existence of a previously uncharacterised antiviral mechanism in higher plants, which may also operate in animals. This defence holds key features of an elaborate immune system, as it is adaptive, mobile and specific. It is also shown, here, that plant viruses have elaborated counter-defensive measures to overcome the host PTGS response, by producing suppressor proteins that target various steps of the silencing mechanism (Chapters 6, 7). One of these factors, the PVX-encoded p25 protein, had been previously characterised as a facilitator of viral cell-to-cell movement. The finding that p25 specifically inhibits the signalling step of PTGS (Chapter 8) provides a new ground for the investigation of virus movement in plants. In chapter 9, the role of PTGS in plants and its suppression by viruses is discussed in the broader context of plant development and biotechnological applications.

Cette thèse est dediée à mes parents, en reconnaissance de tout ce qu'ils ont fait pour moi.

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Abbreviations

Below is an alphabetical list of all abbreviations used in this thesis, excluding SI units and virus acronymes.

acd	accelerated cell death
ATP	adenosine triphosphate
BSA	bovine serum albumen
$CaCl_2$	Calcium chloride
cDNA	complimentary deoxyribonucleic acid
CP	coat protein
CTAB	hexadecyltrimethylammoniumbromide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphate
DPI	days post inoculation
dsRNA	double stranded RNA
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EF	elongation factor
eiF	eukaryotic initiation factor
EMS	ethyl methansulfonate
GFP	green fluorescent protein
GTP	guanosine triphosphate

GUS	β-glucuronidase
kb	kilo-base pairs
KCl	potassium chloride
kD	kilo dalton
L media	luria media
M2	progeny of a mutagenised population
MES	(2-[N-Morpholino]ethanesulfonic acid
min	minute
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
MOPS	3-[2 hydroxyethyl]-piperazine-N'[2ethanesulfonic acid]
MP	movement protein
mRNA	messenger RNA
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
$(NH_4)_2SO_4$	ammonium sulphate
NO	nitric oxide
OD	optical density
PCD	programmed cell death
PCR	polymerase chain reaction
PDR	pathogen derived resistance
PDS	phytoene desaturase

PEG	polyethylene glycol
PIPES	piperazine-N,N'-bis[2-ethane-sulfonic acid]
Qβ	Q-beta phage
RdRp	RNA dependent RNA polymerase
RNA	ribonucleic acid
ROIs	reactive oxygen intermediates
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
sg	sub-genomic
SSC	saline sodium citrate
TAE	tris acetate EDTA
TBE	tris borate EDTA
T-DNA	transferred deoxyribonucleic acid
TE	tris(hydroxymethyl)amino methane and ethylenediaminetetraacetic acid
Tm	temperature of melting
tom 1	tobamovirus multiplication 1
tom1	tobamovirus multiplication 2
UTP	uridine triphosphate
UV	ultra violet
VIGS	virus-induced gene silencing
X-gluc	5-bromo-4-chloro-3-indoyl glucuronide

PUBLICATIONS

Some of the data from Chapter 3 has been published previously as a joint-first authors paper, as:
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Some of the data from Chapter 6 has been published previously as a joint-first authors paper, as:
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Parts of the general discussion in Chapter 9 has been published as a review article as:
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CHAPTER 1

General Introduction

1.1 Transgene-induced gene silencing

Shortly after the discovery of the Ti plasmid of *Agrobacterium tumefaciens*, molecular biologists started to exploit plant transformation to ameliorate or modify agronomic traits such as yield, fruit ripening or disease resistance. Initially, most transformation experiments were in model species belonging to the solanacae family (tomato, potato, tobacco, petunia). This first wave of genetic engineering started in the middle of the eighties and is the origin of the current interest in gene silencing phenomena because, for any given transgene construct, a proportion of the primary transformants did not express the product of the introduced gene, despite its stable integration into the plant genome. At that time, this genetic extinction was regarded as an artefact of transgenesis, and those particular lines were just simply discarded. However, a small community of scientists became interested in the phenomenon and embarked on a systematic analysis in several plant species transformed with several types of constructs.

A major stimulus of this interest was the observation that silenced transgenes could cause the extinction, in *trans*, of homologous, initially highly expressed transgenes (Matzke et al., 1989). In addition, there was the subsequent discovery that endogenous genes could also be targeted by this process (Napoli et al., 1990; van der Krol et al., 1990). This initial

work led to the identification of two classes of gene silencing processes, depending on their effect on transcription of the introduced genes.

1.1.1 Transcriptional gene silencing

1.1.1.1 cis and trans-inactivation

Transcriptional gene silencing (TGS) can be divided into two classes of processes: cis-inactivation and trans-inactivation. Transgenes undergo cis-inactivation when one or multiple copies integrate in or next to a DNA locus that is hypermethylated (Pröls and Meyer, 1992). This phenomenon resembles the position effect variegation (PEV) observed in *Drosophila* when a transcriptionally active gene is brought into contact with heterochromatin through chromosome rearrangement. Spread of heterochromatin into the gene causes random, cell-autonomous and clonal inactivation that appears as variegation (Karpen, 1994). Cis-inactivation in plants can also affect single copy transgenes that are not inserted into hypermethylated DNA (Meyer and Heidmann, 1994). In this case, a difference between the DNA composition of the transgene and that of the surrounding genomic sequences is thought to act as a trigger for inactivation. For instance, this type of TGS was observed when a transgene derived from a monocot was introduced into a dicot, whereas it was not observed if the transgene was isolated from the dicot (Elomaa et al., 1995). Finally, cis-inactivation can also be triggered by transgene repeats that integrate in non methylated DNA. In transgenic *Arabidopsis*, this repeat-induced gene silencing is correlated with increased methylation of the transgene (Assaad et al., 1993).

Transcriptional trans-inactivation occurs when a transcriptionally silenced transgene imposes its silenced state to an unlinked, initially active transgene copy. In some cases, the target transgene itself can acquire the capacity to inactivate other transgene copies that are subsequently introduced through crosses, for instance (Meyer et al., 1993). This is reminiscent of the "paramutation" phenomenon affecting some host genes in maize. Paramutator alleles inhibit the expression of paramutable alleles which themselves become paramutators (Patterson and Chandler, 1995). In other cases of trans-inactivation, the target transgene does not acquire the capacity to inactivate unlinked transgene copies. In several examples, it was shown that this type of trans-inactivation relies solely on promoter sequence homology between the triggering locus and the target (Matzke et al., 1989; Vaucheret, 1993). The promoter region becomes methylated and it is assumed that the modified DNA prevents transcription of the transgene.

1.1.1.2 Possible triggers of TGS

Repeat-induced cis-inactivation and paramutation-like trans-inactivation are both presumed to occur through DNA-DNA pairing, in cis and trans, respectively. Direct physical evidence for DNA-DNA interactions is still lacking in plants, although in fruit flies and fungi there are known examples of homologous DNA sequences pairing within a chromosomal context. For instance, the polytene chromosomes of *Drosophila* in sister chromatids pair at mitosis and occasionally at interphase. Homologous regulatory sequences also interact during the transvection phenomenon in *Drosophila*, in which certain alleles of a given locus, in a heterozygous combination, are able to complement one another, provided that they are juxtaposed by somatic chromosome pairing (Wu and

Morris, 1999). A recent study suggests that transvection also occur in plants (Matzke M et al., 2001). Pairing-dependent modification of DNA sequence duplications also occurs during the sexual cycle of two filamentous fungi, *Neurospora crassa* and *Ascobolus immersus*. In *N. crassa*, the process of RIP (repeat-induced point mutation) is due to duplicated DNA sequences, ranging in size from a few hundred to several thousand base pairs, that incur G-C to A-T transition mutations, following presumed DNA-DNA homologous interactions (Selker, 1999). RIP-modified sequences become substrates for DNA-methyl-transferases, which convert any remaining cytosines to 5-methylcytosine. In *A. immersus*, sequence repeats become heavily methylated and silenced, in a process called MIP (methylation induced premeiotically). During MIP, DNA methylation is transferred interchromosomally at meiosis between paired alleles, through a mechanism related to homologous recombination (Colot et al., 1995). This phenomenon thus provides evidence that DNA-DNA pairing can act as a signal for methylation and TGS.

Recent studies of trans-inactivation operating through promoter homology support the idea that RNA can also act as a trigger for TGS in plants. In several examples, transcriptionaly active transgene loci that are arranged as an inverted repeat encompassing promoter sequences cause consistent and heritable TGS of homologous target loci (Mette et al., 1999) (Mette et al., 2000). In the target transgene locus, DNA methylation is restricted to the promoter region that is homologous to the inverted sequence. Because TGS is not triggered by similar transgenes in which promoter sequences are not part of an inverted repeat, it has been proposed that double-stranded (ds)RNA produced by the silencer locus could act as a diffusible signal for DNA

methylation of unlinked homologous promoter target sequences. In this case, methylation could result from dsRNA-DNA pairing. Strong evidence that dsRNA can direct methylation of transgene sequences in plants (in this particular case, a transcribed region) was provided by Wasseneger and colleagues (Wassenegger et al., 1994). It was shown that a viroid , plant pathogen with a genome composed of circular RNA duplex, can induce de novo methylation of a stably integrated homologous viroid cDNA fragment.

1.1.1.3 mechanism of TGS

Although methylation is often correlated wiht TGS, its role is still unclear. For instance, TGS is not compromised in transgenic plants lacking methyltransferase activity (Mittelsten Sheid et al., 1998). In addition, PEV occurs in adult *Drosophila*, where DNA methylation is lacking (Karpen, 1994). Methylation could constitute one of several possible signals for chromatin modifications or could be simply a bypass product of these modifications. Thus, a change in DNA condensation and not methylation *per se* could be the cause of TGS. Supporting this idea, it has been shown that, in plants, transgenes that are silenced by repeat-induced TGS or through a discrepancy in DNA composition show enhanced resistance to DNAse I and microccocal nuclease treatments, indicating a change in chromatin structure (Ye and Signer, 1996). A direct link between chromatin structure and TGS in plants came from an analysis of the *Arabidopsis ddm1* mutant, which is deficient in methylation and in TGS. DDM1 is indeed a homologue of a component of a chromatin remodelling complex in yeast, thought to induce transcription repression through chromatin condensation (Jeddeloh et al., 1999). Another protein,

SOM1, was also shown to be necessary for TGS, although the function of this factor is currently unknown (Amedeo et al., 2000).

1.1.2 Post-transcriptional gene silencing

Note: this section is deliberately focussed on post-transcriptional gene silencing (PTGS) in plants and will be restricted to work published up to 1998, when this thesis was started. This is to accommodate progressively the results that are presented in the following chapters, so that a general model for the role and mechanism of PTGS emerges eventually in the general discussion. The general discussion will also encompass various aspects of RNA interference, a PTGS-like phenomenon that was discovered in animals in the course of this work. Biochemical and genetic investigation of RNAi have contributed significantly to our current understanding of PTGS in plants.

In PTGS, transcription is not affected, but the steady-state level of RNA is lower than for the nonsilenced transgene. A now classical example of PTGS was provided more than ten years ago in studies of transgenic petunias (Napoli et al., 1990; van der Krol et al., 1990). These plants had been engineered to carry extra copies of the chalcone synthase (CHS) gene, which is involved in flower pigmentation. It was found that a proportion of the transformants did not show the expected deeper purple colour in petals. In contrast, they appeared to have the opposite phenotype: flowers had white petals, with no pigments. Two pioneering studies established that the stability of both the endogenous and transgene CHS RNA was reduced in those lines (Napoli et al., 1990)](van der Krol et al., 1990).

Run-on experiments indicated that none of the corresponding loci had decreased transcription levels, when compared to non-silenced transgenic lines. Importantly, these studies also showed that only RNA with nucleotide-sequence homology to the introduced CHS gene were targeted for post-transcriptional degradation. This process leading to simultaneous extinction of a transgene and its endogenous gene homologue was termed co-suppression. The discovery of co-suppression indicated the existence in plants of a trans-acting, sequence-specific RNA turnover mechanism. Later on, many examples of co-suppression were reported in the literature, involving either transgene-transgene or transgene-endogenous gene combinations reviewed in (Vaucheret et al., 1998). In addition, it was shown that single copy transgenes with no homology to plant genes could trigger PTGS (Elmayan and Vaucheret, 1995). A phenomenon similar to co-suppression, called quelling, was also reported in transgenic *N. crassa* (Cogoni et al., 1996). A collective analysis of these various examples of PTGS indicated that, in all cases, only cytoplasmic RNAs were targeted for degradation, with accumulation of nuclear transcript being unaffected.

1.1.2.1 Triggers of PTGS in transgenic plants

1.1.2.1.1 PTGS mediated by sense transgene contructs

One striking aspect of PTGS mediated by sense-transgenes is its unpredictability, as it usually occurs in a random proportion of the primary transformants. In addition, some progeny of an initially nonsilenced plant may become silenced, and this switch is often influenced by environmental changes or the age of the plant (Palauqui and Vaucheret, 1995). These characteristics indicate that sense-mediated PTGS behaves as an epigenetic

trait. Various factors including ectopic DNA interactions, DNA methylation and transgene expression levels had been proposed as initiators of PTGS triggered by sense transgenes (Vaucheret et al., 1998). However, it is not easy to investigate these factors systematically in transgenic plants. This is partly due to the complex and poorly understood effects exerted by flanking chromosomal DNA on transgene expression (Iglesias et al., 1997). In addition to this position effect, analyses are complicated by the influence of the transformation technique used, which sometimes results in unusual multimeric transgene complexes that are potentially associated with variable amounts of DNA from the transformation vector. Consequently, an analysis of sense-mediated PTGS required the generation of many independent transgenic lines to average the noise in the experimental data. Even though the information gathered from the initial studies was rather biased and often circumstantial, two types of models emerged.

1.1.2.1.1.1 DNA pairing model

The DNA pairing model holds that PTGS initiation relies on ectopic or allelic interactions between coding regions of homologous transgenes. This type of interaction would lead to chromatin modifications that would affect elongation of transgene transcripts, resulting in formation of aberrant RNA (Baulcombe and English, 1996). Aberrant RNA would then be recognised in the cell and would serve as trigger for PTGS, either directly or indirectly. This model was supported by the findings that methylation (and potentially chromatin modification) of the transgene coding region is often, although not always, associated with PTGS (Baulcombe and English, 1996). In addition, it had been demonstrated that MIP in *A.immersus* can cause an arrest of transcription elongation, leading to prematurely truncated (and therefore aberrant) RNA species(Barry et al., 1993).

1.1.2.1.1.2 RNA threshold model

The RNA threshold model was developed from the observation that PTGS is often triggered by transgene constructs that are driven by strong promoters, such as the cauliflower mosaic virus (CaMV) 35S promoter. The effect of transgene dosage on initiation of PTGS, as well as the recovery observations of Lindbo and Dougherty (section 1.2.4) are also consistent with the RNA threshold model (Elmayan and Vaucheret, 1996) (Lindbo et al., 1993).

Another reason to invoke this type of models was the demonstration that hemizygous, single copy transgenes (hence, with no potential to pair with homologous DNA) could trigger PTGS (Elmayan and Vaucheret, 1996). The RNA threshold model holds that

PTGS occurs as a cellular response to RNA accumulating over a critical limit in the cell. DNA pairing and RNA threshold models are not mutually exclusive. For example, PTGS could be triggered by over-accumulation of a subset of aberrant RNA resulting from DNA-DNA interactions between transgene copies.

1.1.2.1.2 PTGS mediated by inverted repeats and hairpin constructs

In contrast to the random nature of sense-mediated silencing, it appears that PTGS is much more consistently activated by transgene constructs that have the potential to produce dsRNA. The first indication came from analysis of PTGS in transgenic petunia. It was found that in a significant proportion of plants exhibiting PTGS, transgene loci were organised as inverted repeats, and thus had the potential to form dsRNA-like structures through intramolecular base-pairing (Stam et al., 1998). Subsequently, it was shown that an inverted repeat that was deliberately engineered in the 5' UTR of a transgene encoding the ethylene biosynthetic enzyne ACC oxydase caused almost 100% cosuppression in tomato transformants (Hamilton et al., 1998). The work of Waterhouse and co-workers further strengthened the idea that dsRNA is a potent inducer of PTGS in plants (Waterhouse et al., 1998). The authors introduced, into separate tobacco plants, gene constructs expressing the sense polarity or the antisense polarity of the coding sequence of a transgene. For these control constructs, a low frequency of primary transformants exhibited PTGS, whereas, in contrast, there was a high frequency of PTGS in plants were both constructs had been brought together through crossing. An even higher proportion of silencing lines was obtained when plants were directly transformed with tandem gene constructs linked in cis, in which one gene was for expression of sense

orientation sequence and the other for expression of the antisense sequence (Waterhouse et al., 1998). Consistent silencing (up to 100%) is achieved in various plant species by transformation of stem-loop constructs in which almost perfect RNA duplexes can be formed by intra-molecular pairing of coding regions (Chuang and Meyerowitz, 2000) (Smith et al., 2000). Altogether, these findings indicate that silencing activated by transgene loci producing dsRNA is inherited as a Mendelian trait, in contrast to the epigenetic nature of silencing triggered by sense constructs. This led to the suggestion that dsRNA is a critical and probably essential intermediate in the development of PTGS in plants.

1.1.2.2 Mechanism of transgene-induced PTGS in plants

At the time this thesis was initiated, none of the cellular factors required for PTGS in plants had been isolated. However, several *Arabidopsis* mutants impaired in sense-transgene mediated silencing (*suppressors of gene silencing* mutants, *sgs*) had been described (Elmayan et al., 1998). Several mutants impaired in quelling (*quelling defective* mutants, *qde*) were also isolated in *N.crassa* (Cogoni and Macino, 1997). Our laboratory also embarked on a genetic approach of PTGS in *Arabidopsis*. Despite a lack of information about the components of PTGS, several predictions were made concerning the mechanism.

First, if dsRNA is a prerequisite for PTGS, then the question arises as to how this molecule is synthesised in sense transgene-mediated PTGS. As PTGS was predicted to be directly or indirectly triggered and mediated by RNAs, this step was proposed to occur at the level of RNA and to involve the activity of an RNA-directed-RNA-polymerase

(RdRp) (Dougherty and Parks, 1995). Several models predicted that the putative aberrant RNA produced from sense transgene loci (as proposed in the ectopic pairing model, see above) would constitute the template for the RdRp, leading to formation of dsRNA. This suggestion was supported by the identification of an endogenous RdRp activity in tomato and the subsequent purification of the protein (Schiebel et al., 1998). In addition, the fact that PTGS is an extremely potent process capable of eliminating RNA produced from highly transcribed loci led to the early suggestion that a host RdRp activity could be involved in amplification of the silencing response (Dougherty and Parks, 1995).

A second likely component of the PTGS mechanism is a specificity determinant allowing RNAs to be targeted if they are similar to the silencing transgene. Because sense transgene RNA can cause sense RNA degradation, it had been proposed that antisense RNA may constitute the PTGS specificity determinant. By forming a duplex with the target RNA, this putative molecule would promote its degradation or interfere with its translation. If these hypothetical antisense RNA molecules are of similar size to typical mRNAs, they would have been readily detected by conventional RNA analyses. However, there had been no report of such molecule being detected consistently in plants exhibiting PTGS, suggesting that if the antisense RNA existed, they would be too short for easy detection. This reasoning prompted investigation, in our lab, of PTGS-specific, low molecular weight antisense RNA species (Andrew Hamilton)

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A third component that was expected to be part of the PTGS mechanism is a nuclease that would ensure degradation of homologous mRNA, probably in association with the specificity determinant of PTGS.

1.2 Transgenes, PTGS and antiviral protection

In 1985 Sanford and Johnston proposed the concept of pathogen-derived-resistance (PDR), whereby interference with the biology of a pathogen would result from expression, in a susceptible organism, of proteins derived from this pathogen (Sanford and Johnston, 1985). This concept predicted that the interference would be most effective when conferred by dominant negative forms of pathogen-derived proteins.

1.2.1 Protein-mediated resistance

Shortly after the proposal of Sanford and Johnston, the concept of PDR was tested in the context of plant virus-interaction: the effect of transgenic expression of viral proteins, modified or not, was investigated. Possible targets of PDR included factors that are necessary for protection, movement and replication of the viral nucleic acid.

1.2.1.1 Coat-protein-mediated resistance

In 1986, Powell-Abel and colleagues published the first example of coat-protein-mediated resistance (Powell et al., 1986). Transgenic tobacco plants that expressed constitutively the coat protein (CP) of tobacco mosaic virus (TMV) were shown to exhibit some level of resistance against TMV. The characteristics of this resistance were the following:

① partial: in the best cases, a strong attenuation of symptoms was observed. However, even those plants eventually developed the disease,

② broad spectrum: the resistance was effective against several types of tobamoviruses, which is the family to which TMV belongs,

③ dependent on high levels of expression of a functional CP,

④ effective on assembled viral particles, but not against an RNA inoculum.

Several experiments further suggested that the resistance was due to an inhibition of particle uncoating as well as delayed cell-to-cell movement (the coat protein is necessary for tobamoviruses cell-to-cell movement). Later on, many similar examples were described in the literature, involving various plant-virus combinations reviewed in (Baulcombe, 1996).

1.2.1.2 Movement-Protein-mediated resistance

The first case of movement-protein-mediated-resistance was reported in 1993, again in the case of TMV (Lapidot et al., 1993). Transgenic tobacco that constitutively expressed a mutated form of the TMV cell-to-cell movement protein (MP) exhibited some resistance against TMV. Resistance had the following attributes:

① partial,

② broad spectrum: the resistance was effective against several tobamoviruses as well as several cucumo- and potexviruses,

③ dependent on high levels of expression of a dysfunctional protein engineered with a dominant-negative mutation

It was proposed that the resistance resulted from competition of the mutated MP for putative plasmodesmata docking proteins. Several other examples of MP-mediated resistance were subsequently reported reviewed in (Baulcombe, 1996).

1.2.1.3 Replicase-mediated resistance

The replicase of viruses being the essential component of their biology, strategies aimed at interfering directly with this function were investigated in plants. Such approaches were mostly inspired by previous studies in *E.coli*. Its was shown in those studies that recombinant *E.coli* cells expressing an altered form of the Qß phage replicase were resistant to the phage. It was established that the altered replicase was competing with the native form for replication sites of the pathogen genome (Inokuchi and Hirashima, 1987). In 1993, it was shown in our laboratory that some tobacco transformants expressing constitutively a mutated form of the replicase of potato virus X (PVX) were resistant to PVX (Longstaff et al., 1993). This resistance had the following features:

① extreme: there was no symptom developing on inoculated plants, and accumulation of PVX in protoplast was drastically reduced,

② strain-specific: the resistance was operative only against PVX strains sharing high nucleotide sequence homology with the strain from which the engineered replicase was derived.

These features clearly contrasted with the previously characterised examples of protein-mediated resistance.

1.2.2 Homology-dependent resistance: viruses as targets of PTGS

Following the work on replicase-mediated resistance to PVX, a new set of replicase constructs was engineered. These constructs carried a frameshift mutation in the replicase ORF, preventing expression of a functional protein. Tobacco transformants were obtained, and some lines exhibited the same type of extreme resistance as mentioned

above. Because of the introduced frameshift mutation in the replicase ORF, it was difficult to explain the resistance as a protein-mediated effect (Mueller et al., 1995).

The surprise came when further analysis revealed that, in the resistant lines, the replicase mRNA was undetectable in Northern blot analysis. However, high levels of replicase RNA were readily detected in the susceptible lines (Mueller et al., 1995). This inverse correlation between resistance and RNA levels combined to the nucleotide-sequence-specificity of the effect were suggestive of a silencing-related mechanism. Further run-on analysis confirmed this hypothesis, and showed that the replicase RNA was targeted by PTGS. The characteristics of the resistance indicated that the PTGS mechanism was effective against a challenged viral RNA accumulating in the cytoplasm.

At the same time, Lindbo and co-workers demonstrated a similar effect in plants engineered with the CP of tobacco etch virus (TEV), a potyvirus. Plants exhibiting PTGS of the CP RNA were immune to challenged TEV (Lindbo and Dougherty, 1992). Both studies indicated that viruses carrying sequence-homology to a silenced nuclear transgene were targeted by the PTGS mechanism, thus defining the concept of homology-dependent-resistance (HDR). This concept proved to be widely applicable to many plant-virus combinations, and have been recently successfully exploited in the field. For instance, this strategy was used in Hawai, to confer resistance to papaya trees against the papaya ringspot virus (PRSV) (Fitch et al., 1992). In a large-scale trial, a transgenic line called "Rainbow" showed remarkable levels of resistance in an area that had previously been totally devastated by the virus.

1.2.3 Homology-dependent resistance mediated by transgenes of non viral origin

It was subsequently shown that non-viral sequences could mediate HDR against recombinant viruses. For example, plants exhibiting PTGS of a GUS transgene were resistant to a PVX vector engineered with GUS sequences (English et al., 1996). However, these plants were not resistant against PVX carrying an insert of the GFP gene. In addition, control plants in which a GUS transgene was silenced at the transcriptional level were not resistant to PVX-GUS (English et al., 1996). These experiments thus established a strict correlation between PTGS and HDR, by showing that viruses could be targets of the PTGS mechanism provided they shared sequence-homology to a silenced transgene.

1.2.4 Viruses as potential inducers of PTGS

Investigation into the mechanism of PTGS on the one hand, and plant resistance to viruses on the other, began to converge with studies of the mechanism of "recovery" in virus-infected transgenic plants. This phenomenon was discovered in the course of experiments on tobacco plants carrying a highly expressed transgene encoding the CP of TEV (Lindbo et al., 1993). When these transgenic lines were infected with TEV, symptoms initially appeared but the new growth generated after infection was found to be specifically resistant to a secondary challenged with TEV. In the new emerging tissues, the transgene CP mRNA was degraded at the post-transcriptional level. The authors proposed that infection of TEV activated PTGS of the CP transgene, such that it subsequently conferred HDR against the inducing virus (Lindbo et al., 1993).

Further insight into the potential relationship between viruses and PTGS was provided by the work of Kumagai and colleagues (Kumagai et al., 1995). The authors attempted to transiently decrease the levels of phytoene desaturase (*PDS*) gene in tobacco by infecting plants with a recombinant TMV vector engineered with an antisense fragment of the *PDS* cDNA. The goal was to manipulate the cellular content of PDS, an enzyme involved in production of carotenoids, a class of photoprotective pigments. Upon infection with the recombinant TMV, the plants developed symptoms of photo-bleaching, similar to the phenotype of plants deprived of carotenoids. Further analysis showed that the PDS mRNA levels were reduced in virus-infected cells and that this effect was nucleotide-sequence specific (Kumagai et al., 1995). The authors interpreted these results as a consequence of antisense inhibition due to formation of hybrids between viral and endogenous PDS RNAs, preventing translation of the endogenous transcript. As a similar photo-bleaching effect was observed when the PDS cDNA fragment was introduced in the sense orientation in the TMV vector, it was proposed that such hybrids were also formed with the negative strand RNA of the virus.

There was, however, an alternative explanation for these results. It was possible that infection with the recombinant TMV had triggered a form of sequence-specific RNA turn-over mechanism akin to PTGS of transgenes. If this process was naturally activated by and targeted against the TMV genome, independently of host nuclear gene homology, the sequence-specificity of PTGS would have derived from the recombinant TMV RNA and thus both the viral and the host PDS transcripts would have been targeted for degradation, leading to a photo-bleaching phenotype.

Further work supported this suggestion by showing that virus infection itself is sufficient to induce a PTGS-like response in the absence of sequence homology between viruses and host nuclear genes. For instance, new emerging leaves of wild-type tobacco plants that are infected with tomato black ring virus (TBRV) recover in the same way as the TEV-CP transgenic tobacco recovered from TEV infection (Ratcliff et al., 1997). It was demonstrated that the TBRV-recovered tissues exhibited sequence-specific resistance against secondary challenged with the virus, or against a PVX vector carrying fragments of the TBRV genome (Ratcliff et al., 1997). A second example of this phenomenon was provided by an analysis of Cauliflower mosaic virus (CaMV)-recovered leaves in wild-type kohlrabi and oilseed rape. In these tissues, recovery was also associated with the specific loss of viral RNA (Covey et al., 1997).

1.3 Objectives of this study

The aims of this study are: (i) to exploit plant viruses as RNA inducers of sense-mediated PTGS in order to better understand the underlying mechanism; (ii) to investigate the potential role of PTGS as a natural antiviral defence mechanism and (iii) to elucidate how viruses accommodate the plant gene silencing defence.

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CHAPTER 2

Material and methods

General Comments

This chapter describes the experimental protocols used throughout the research. Most procedures are those described by either Sambrook *et al.* (Sambrook et al., 1989) or Ausubel *et al* (Ausubel et al., 1990). Modifications are indicated in the appropriate sections. Buffers and solutions were prepared according to Sambrook *et al.* with deionised water from a reverse osmosis water purification system (ELGA), and were either sterilised by autoclaving (15 lb/in^2 at 121°C for 20 min) or by filtration. Ethanol precipitation and phenol/chloroform extraction of nucleic acids were performed according to Sambrook *et al.* Spin dialysis was used to remove salts and low molecular weight contaminants from nucleic acid preparations as described by Murphy and Kavanagh . Commercial enzymes and kits were used in accordance with their manufacturers' instructions.

Sample volumes up to 1.5 ml were centrifuged in a Microspin 24S centrifuge (Sorvall Instruments). Sample volumes from 1.5-50 ml were centrifuged in either an MSE 2000 bench top centrifuge (Mistral) or an RC5B refrigerated superspeed centrifuge (Du Pont Instruments). Sample volumes over 50 ml were centrifuged in an RC3B Refrigerated centrifuge (Du Pont Instruments).

2.1 Bacteria

2.1.1 Strains

The strains of *Escherichia coli* listed below were used for cloning experiments and plasmid propagation:

DH5α (Hanahan, 1983), F', ϕ80d*lac*ZΔM15, Δ(*lacZ*YA-*argF*)U169, *RecA*1, *endA*1, *hsd*R17, *supE*44,*thi*-1, *gyrA*, *relA*1.

DH10B (Grant, et al., 1990) F^-, *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80d*lac* ZΔM15, Δ*lac*X74, *deo*R, *recA*1, *end*A1, *ara*D139, Δ(*ara, leu*)7697, *gal*U, *gal*Kl^-, *rps*L, *nup*G.

The *Agrobacterium tumefaciens* strain used for transient gene expression *in planta* was UIA143 (Farrand et al., 1989), carrying tetracycline resistance and extra copies of *vir G*, *vir E1*, and *vir E2* on the plasmid pCH32.

2.1.2 Growth media

E. coli and *A. tumefaciens* were grown on either Luria (L) broth (1% Difco Bacto-triptone, 0.5% Yeast extract, 0.5% NaCl, 0.1% D-glucose), or L medium (L broth containing 1.5 % bacto-agar). Depending on the plasmid, growth media for transformed *E.coli* was supplemented with tetracycline, carbenicillin or kanamycin according to Sambrook *et al.* (Sambrook et al., 1989). Growth media for transformed *A. tumefaciens* was supplemented with 5μg/ml tetracycline, or 50 μg/ml kanamycin. When α-

complementation was required for selection of *E.coli*, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and IPTG (isopropylthio-β-D-galactoside) at concentrations of 0.8 mg/ml and 0.145 mg/ml respectively, were also included. When *A. tumefaciens* was prepared for transient gene expression *in planta*, L broth was supplemented with 10 mM 2-[N-Morpholino]ethanesulfonic acid (MES) pH 5.6.

All *E.coli* was grown at 37°c, while all *A. tumefaciens* was grown at 28°c. Plates were kept in an incubator (Leec); liquid cultures were grown in an orbital shaker at 300 rpm (New Brunswick Scientific).

2.1.3 Transformation

2.1.3.1 Hanahan's basic method

A fresh culture of *E. coli* (100 ml) at an OD_{600} between 0.4 and 0.6 was cooled on ice for 10 min. The cells were recovered by centrifugation at 7000 g at 4 ^{o}C for 5 min. The pellet was resuspended in 20 ml of 0.1 M $MgCl_2$ and stored on ice for 20 min. The bacteria were harvested by centrifugation at 7000 g at 4^{o}C for 5 min and resuspended in 2 ml of 0.1 M $CaCl_2$ and 15 % glycerol. The competent cells were stored at -70^{o}C in 100 µl aliquots.

For transformation, DNA was mixed with 100 µl competent cells, incubated on ice for 30 min and heat shocked at 42 ^{o}C for 1.5 min. The suspension was then mixed with 1 ml LB media and incubated in a shaking incubator at 37 ^{o}C for 45 min. Transformed cells were selected on L agar supplemented with appropriate antibiotics.

2.1.3.2 Electroporation of E. coli and A. tumefaciens

E. coli DH10B was made electrocompetent as follows: 350 ml of SOB medium (2 % Bacto tryptone, 0.5 % Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl) in a 2 l flask was inoculated with 0.5 ml of an overnight culture of bacteria and incubated for 3-4 hours at 37 °C with vigorous shaking. When the OD_{550} reached 0.7, the culture was chilled on ice for 30 min. The cells were harvested at 4000 g for 15 min at 4 °C, washed twice with 350 ml 10 % glycerol, and resuspended to a final volume of 1.4 ml in ice cold 10 % glycerol. The cells were used immediately or stored at -70 °C in 100 µl aliquots.

A. tumefaciens UIA143 was made electrocompetent as follows: 350 ml of L broth was inoculated with 0.5 ml of an overnight culture of bacteria and grown to saturation overnight in the presence of 5 µg/ml tetracycline. The cells were pelleted at 4000 rpm for 15 min at 4°c, washed four times with 350 ml of SDW, and resuspended in 1.4 ml of 10% glycerol. Cells were used immediately or stored at -70 °C in 100 µl aliquots.

0.5-3 µl of a ligation which had previously been spin dialysed was added to 20 µl of electrocompetent cells. The electroporation was performed using the GIBCO BRL electroporator (Cell-porator and Voltage Booster) in a pre-chilled 0.15 cm cuvette according to the manufacturers conditions (Capacitance: 330 µF, Resistance: 4000 Ω, Voltage: 400 V, Impedance: low Ω, charge rate fast). After electroporation cells were

transferred to 1 ml of L broth and incubated for 1 hour with gentle shaking (100 rpm). The transformed cells were selected on L plates supplemented with antibiotic.

2.1.4 Selective enrichment of Agrobacterium from plant tissues

The selective enrichment assay for *Agrobacterium* (Chapter 4) was as described (Matzk et al., 1996). For these experiments systemic silencing (section 2.9.3) was iniated in one leaf only, while the rest of the plant was covered with saran wrapping paper to minimise contamination. The enrichment was from sap extracts of tissue showing suppression of GFP. In ten samples, the selective enrichment procedure detected *A.tumefaciens* in 10^{-12}-fold dilutions of infiltrated leaf extracts. However, in more than forty samples from systemic tissues (including stems and apexes) exhibiting full or partial silencing of GFP, the infiltrated *A.tumefaciens* was not detected, even in undiluted samples.

2.2 Plasmid DNA preparation

2.2.1 Small-scale isolation of plasmid DNA

This method is a modification of that described by Birnboim and Doly. A 5 ml overnight culture of bacteria, grown in L broth supplemented with appropriate antibiotics, was centrifuged at 3000 rpm at 4 ^{o}C for 15 min. The pellet was suspended in 100 µl of Solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8). The bacteria were lysed with 200 µl of Solution II (0.2 N NaOH, 1 % SDS) at room temperature for 5 min. The pH of the mixture was neutralised by addition of 150 µl of Solution III (3 M potassium, 5 M acetate). The mixture was incubated on ice for 5 min

and cleared by centrifugation at 12000 rpm for 5 min. The supernatant was extracted once with phenol/chloroform and the nucleic acids were precipitated with 0.6 volumes of isopropanol. The pellet was washed with 1 ml 70 % ethanol, dried in the vacuum centrifuge for 2 min and resuspended in 50 µl TE containing RNAase A at 10 µg/ml.

2.2.2 Large-scale isolation of plasmid DNA

High quality DNA for cloning, *in vitro* RNA transcription, and sequencing was routinely obtained using the Tip-100 DNA preparation kit (QIAGEN) according to the manufacturer's instructions. One phenol/chloroform extraction followed by spin dialysis was performed on the recovered DNA to ensure purity. For high copy plasmids the procedure yields approximately 100 µg of plasmid DNA from a 30ml bacterial culture.

2.3 Manipulation of Nucleic Acids

2.3.1 General

All routine enzymatic manipulations of DNA including restriction enzyme digests, dephosphorylation, ligation and the preparation of blunt-end DNA with T4 DNA polymerase, were performed according to the manufacturers instructions except for the modifications cited below. When the buffer of the reaction needed to be changed, the solution was dialysed through a sepharose CL-6B column as described by Murphy and Kavanagh (Murphy and Kavanagh, 1988).

2.3.2 DNA ligation reactions

Plasmid vectors (50 ng) were ligated to insert fragments at a molar mass ratio of 1:3 in 10 µl of buffer (66 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM DTE, 1 mM ATP, pH 7.5) with 5 units of T4 DNA ligase (Boehringer) for 2 hours at 23 °C, or overnight at 16°c.

2.3.3 Blunting ends with T4 DNA polymerase

Ligation of DNA fragments with incompatible ends was preceded by exonuclease digestion with T4 DNA polymerase. Gel purified PCR products or DNA fragments from restriction digests were blunted using 5 units of T4 DNA polymerase in 1x T4 DNA polymerase buffer (GIBCO BRL) containing 0.3 mM dNTP at 12°c for 15 min. Reactions were stopped by phenol/chloroform extraction then spin dialysed. This DNA was used directly for ligation.

2.3.4 Dephosphorylation of DNA

DNA was dephosphorylated using heat-labile phosphatase. 1 µg of linearised plasmid was dephosphorylated at 30°C for one hour in buffer containing 33 mM Tris-acetate pH 7.8, 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM DTT, 5 mM $CaCl_2$ and 1 unit of phosphatase (Epicentre, USA). The phosphatase was inactivated at 65 °C for 30 min followed by phenol/chloroform extraction and spin dialysis.

2.3.5 In vitro transcription reactions

PVX and TMV *in vitro* RNA transcripts were produced using T7 DNA dependent RNA polymerase. 2.5 µg of linearized plasmid was transcribed at 37 ^{o}C for 25 min in buffer containing 40 mM Tris-HCl pH 8, 8 mM $MgCl_2$, 2 mM spermidine, 25 mM NaCl, 5 mM DTT, 40 units RNase inhibitor, 2 mM of each ATP, CTP and UTP, 0.2 mM GTP, 0.5 mM m7G(5')ppp(5')G and 100 units of T7 RNA polymerase. The concentration of GTP was then adjusted to 2 mM and the reaction continued for a further 40 min. A 1µl aliquot was run at 10 v/cm on a 1% agarose TBE gel.

2.3.6 DNA sequencing

Sequencing reactions were carried out using the Big Dye kit according to the manufacturer's protocol (Perkin Almer) except that the final volume was changed from 20 µl to 10 µl. Sequencing reactions were precipitated by adding 3.5 µl of 7.5 M ammonium acetate and 35 µl absolute ethanol. The samples were run on acrylamide sequencing gels and analysed by ABI software. Software in the DNASTAR package was employed to edit and align sequences.

2.3.7 Isolation of plant genomic DNA

Leaf tissues (5 g) were ground in a pestle and mortar with liquid nitrogen. The powder was suspended in 23 ml of preheated extraction buffer (100 mM Tris-HCl pH 8, 50 mM EDTA pH 8, 500 mM NaCl, 1.25 % w/v SDS, 8.3 mM NaOH, 0.38 % sodium bisulfite and 0.38 % sodium diethyldithiocarbamate) and incubated at 65 ^{o}C for 15 min. The

suspension was mixed with 7.1 ml of 5 M potassium acetate, incubated on ice for 20 min and centrifuged at 3000 rpm at 4 °C for 10 min. The supernatant was filtered through miracloth into a fresh tube, mixed with 20 ml of isopropanol and centrifuged at 3000 rpm for 10 min. The pellet was washed with 2 ml of 80% ethanol, resuspended in 2 ml of H_2O, mixed with 2 ml of 2x CTAB (200 mM Tris-HCl pH 8, 50 mM EDTA, 2 M NaCl and 2 % CTAB) and incubated at 65 °C for 15 min. The CTAB caused precipitation of protein and polysaccharides and these were removed by extraction with 4 ml chloroform. The supernatant was mixed with an equal volume of isopropanol and the nucleic acid was hooked out with a heat-sealed Pasteur pipette. The nucleic acid was resuspended in 2 ml SDW. A further two rounds of CTAB and chloroform extraction, followed by isopropanol precipitation were carried out before the DNA was finally washed in 1 ml of 80% ethanol and resuspended in 200-400 µl SDW.

2.3.8 Isolation of plant total RNA

2.3.8.1 Isolation of total high molecular weight RNA

Total RNA was extracted according to Devic *et al.* (Devic et al., 1990). 5 g of leaf tissue was ground in a pestle and mortar with liquid nitrogen. The powdered material was dispersed in 5 ml of homogenisation buffer (0.1 M NaCl, 2 % SDS, 10 mM EDTA and 50 mM Tris-HCl pH 9), mixed with 5 ml phenol/chloroform and centrifuged at 3000 rpm for 10 min. The supernatant was further extracted with phenol/chloroform and once with chloroform. The nucleic acid was precipitated with 0.6 volumes of isopropanol, washed in 1 ml of 70% ethanol, vacuum dried, and resuspended in 50-100 µl of SDW.

Alternatively, extraction of total RNA was by trireagent (SIGMA), according to the manufacturer's instruction. When total RNA was to be used for 21-23nt RNAs analysis, precipitation was in 100% ethanol for 2 hours at -70°C, instead of isopropanol. In some instances, total RNA extraction was also performed with Tri-Reagent (SIGMA), acvcording to the manufacturer's instructions.

2.3.8.2 Isolation of low molecular weight (21-23nt) RNA

Following extraction of total plant RNA and precipitation in 100% ethanol, the pellet was dissolved in 700 µl of SDW by heating for 5 min at 65°C.100 µl of 5M NaCl was added with 200µl of polyethylen glycol (PEG) 8000. The mix was left on ice for 30 min and subsequently spun in a microfuge at ≥ 5000 rpm for 15 min. The supernatant (i.e. small RNA fraction) was then added three volumes of 100% ethanol and precipitated for at least one hour at -20°C (usually over-night). Centrifugation was carried out at 4000 rpm in blue-cap 15ml Falcon tubes, and the pellet was then resuspended in 200-500 µl of 50% formamide (SIGMA).

2.3.9 Polymerase Chain Reaction Based Techniques

2.3.9.1 Polymerase chain reaction

Polymerase chain reactions (PCR) were routinely performed using 1 to 500 ng of template DNA. The reactions contained: 10 mM Tris-HCl pH 8.3, 2.5 mM $MgCl_2$, 50 mM KCl, 0.05 % Nonidet P-40, 0.2 mM dNTP, 0.4 µM of each primer and 2 to 10 units

Taq DNA polymerase. When blunt ended PCR products were required, reactions were performed with *Pfu* polymerase (STRATAGENE) in a buffer containing, 10 mM $(NH4)_2SO_4$, 20 mM Tris-Cl pH 8.75, 2 mM $MgSO_4$, 0.1% Triton®X-100 and 100 µg/ml bovine serium albumen (BSA). The reaction volume varied between 10 and 200 µl. Cycling conditions were optimised for each polymerase, the primer pair and the length of the PCR product. For reactions using *Taq* polymerase an extension period based on 1 minute per 1 kb was used; for reactions using *Pfu* polymerase this was increased to 2 minutes per 1 kb. PCR was performed in either a DNA thermal cycler model 480 (Perkin Elmer), or in a PTC-200 (MJ Research). The oligonucleotides were synthesised on a Nucleic Acid Synthesis System (Millipore). Following synthesis, oligonucleotides were removed from the support columns with ammonium hydroxide for 15 min at 55 ^oC according to the manufacturers instructions. Oligonucleotides were precipitated with 0.1 volumes of 3M sodium acetate and 3 volumes of 100% ethanol before being resuspended in water at a concentration of 10 µM.

2.3.9.2 Colony PCR

Colony PCR was used as tool for rapid screening of recombinant plasmids during cloning. The PCR was performed as described above except that the DNA template was substituted with bacterial cells, and the thermal cycling conditions were preceded by an additional step of 94°c for 1 min.

2.4 Gel Electrophoresis of Nucleic Acids

2.4.1 Non-denaturing gels

DNA and RNA were electrophoresed in submarine agarose gels containing 1x TBE buffer (90 mM Tris-borate, 2 mM EDTA), 0.1 μg/ml ethidium bromide and 0.7-3 % agarose, in 1x TBE buffer at 10 V/cm. For low molecular weight DNA Nusieve GTG agarose (FMC) was used. If high resolution agarose gels were necessary, such as for Southern blotting procedures, the TBE was substituted with TAE (40 mM Tris-acetate, 2 mM EDTA) and the gel was electrophoresed overnight at 2 V/cm. The 1 kb DNA ladder (GIBCO-BRL) was used as a molecular size marker.

2.4.2 Formaldehyde denaturing gels

RNA samples were mixed with 3 volumes of loading buffer (1 ml formamide [GIBCO-BRL], 0.35 ml of 37 % formaldehyde, 0.1 ml of 10x MOPS/EDTA buffer [50 mM sodium acetate, 0.2 M MOPS pH 7 and 10 mM EDTA], 3.5 mg bromophenol blue, and 10 μg/ml ethidium bromide) and incubated for 10 min at 70 ^{o}C. The samples were then loaded onto a 1 % agarose gel containing 1.9 % formaldehyde and 1x MOPS/EDTA buffer and electrophoresed at 8 V/cm in 1x MOPS/EDTA buffer. The 0.24-9.5 kb RNA ladder (GIBCO-BRL) was used as a molecular size marker.

2.4.3 high percentage acrylamide-urea denaturing gels

RNA samples were mixed with six volumes of loading buffer (solution 2xTBE, 40% sucrose, 0.1% bromophenol blue) and incubated for 10 min at 70 °C. The samples were

then loaded onto a 15% acrylamide-urea gel containing 42g of urea, 37ml of 40% acrylamide-Bis acrylamide 19:1 (Severn Biotech limited), 5 ml of 10X TBE, 700 µl ammonium persulfate (APS) and 35 µl of N, N, N', N'-tetramethylethylenediamine (TEMED). The gel was set on a vertical electrophoresis system from Biorad (PROTEAN II XL Cell), according to the manufacturer's instruction.

2.5 Radiolabelling of Nucleic Acids

2.5.1 Random priming

DNA fragments were labelled using the Oligolabelling Kit according to the manufacturers instructions (Pharmacia). The radiolabelled DNA was spin dialysed through sepharose CL-6B spin columns, denatured by heating to 95 ^{o}C for 2 min, cooled on ice for 1 min and added to the hybridisation solution.

For GFP RNA analysis, the probe used for hybridization was either the cDNA corresponding to the entire GFP5 open reading frame (Haseloff et al., 1997) or a 354-bp 3' terminal fragment of the GFP5 cDNA (fragment referred to as "P" fragment).

2.6 Hybridisation of Nucleic Acid

2.6.1 Southern hybridisation

Restriction digests of plant genomic DNA (5 to 10 µg) were fractionated on 0.8 % agarose gel (see section 2.4.1). The DNA was partially depurinated by soaking the gel in

250 mM HCl for 15 min with gentle shaking. DNA was denatured by soaking the gel in 0.5 M NaOH, 1.5 M NaCl for 30 min and neutralised by soaking in 0.5 M Tris-HCl pH 7.2, 1.5 M NaCl and 1 mM EDTA for 15 min twice. DNA was transferred to Hybond-N^+ membrane (Amersham) by capillary blotting overnight using 20x SSC as the transfer buffer. After transfer, the membrane was rinsed with 2X SSC.

The prehybridisation, hybridisation and washing were performed in glass tubes in a commercial hybridisation oven (Techne). Filters were prehybridised for 2 hours before overnight hybridisation with the radioactive probe in solution containing 50 % formamide, 4x SSC (20x SSC is: 3 M NaCl, 0.5 M sodium citrate pH 7), 5x Denhardt's (50x Denhardt's solution is: 1 % ficoll (400), 1 % polyvinylpyrolidone (360), 1 % bovine serum albumin), 5 % dextran sulphate, 0.5 % SDS and 0.1 mg/ml salmon sperm DNA. The hybridisation was carried out at 42°c. The filters were washed at low stringency in 4x SSC, 0.5 % SDS for 1 hour at 42 °C, or at high stringency in 0.1x SSC and 0.1% SDS at 65 °C for 1 hour.

To estimate the weight of plasmid DNA that is equivalent to one copy per genome the following formula was used;

$$\frac{\text{mass of genomic DNA}}{\text{length of genome}} = \frac{\text{mass of plasmid DNA}}{\text{length plasmid}}$$

For example, to estimate the mass of a 10 kb control plasmid that is equivalent to one copy in 10 μg of a of 1.5×10^9 bp genome:

$$\frac{10^{-6}\text{g}}{1.5 \times 10^9 \text{ bp}} = \frac{\text{mass of plasmid DNA}}{10^4 \text{ bp}}$$

or mass of plasmid DNA = $10^4 \times \frac{10^{-6}}{1.5 \times 10^9} = 6.6 \times 10^{-12}$ g, or approximately 6 pg

The concentration of plasmid stock solutions was estimated by comparative ethidium bromide staining of electrophoresed plasmid and quantitative control DNA (Perkin Elmer). Plasmid solutions were then sequentially diluted in 0.1 μg/μl salmon sperm DNA

2.6.2 Northern hybridisation

2.6.2.1 Northern hybridisation of high molecular weight RNA

RNA (2-10 μg) was run on a denaturing agarose gel (see section 2.4.2). RNA was transferred to Hybond-N membrane (Amersham) by capillary blotting for 8-12 hours in 20x SSC. RNA was cross-linked to the membrane using the Stratalinker Apparatus 2400 (Stratagene). The prehybridisation, hybridisation and washing were performed in glass tubes in a commercial hybridisation oven (Techne). Filters were prehybridised for 2 hours before overnight hybridisation with the radioactive probe in solution containing 50 % formamide, 5x SSPE 0.5% SDS, 5X Denhardt's solution (Sambrook et al., 1989), and 0.1 mg/ml salmon sperm DNA. The hybridisation was carried out at 60 ^{o}C for RNA probes

and at 42 °C for DNA probes. The filters were washed three times in 5x SSC, 0.1 % SDS and three times in 0.1 x SSC, 0.1 % SDS at 65 °C; 15 min for each washing.

2.6.2.2 Northern hybridisation of low molecular weigh RNA

RNA (2-10 μg) was run on a denaturing agarose gel (see section 2.4.2). RNA was transferred to Hybond-N membrane (Amersham) by capillary blotting for 8-12 hours in 20x SSC. RNA was cross-linked to the membrane using the Stratalinker Apparatus 2400 (Stratagene). The prehybridisation, hybridisation and washing were performed in glass tubes in a commercial hybridisation oven (Techne). Filters were prehybridised for 2 hours before overnight hybridisation with the radioactive probe in solution containing 50 % formamide, 5x SSPE 0.5% SDS, 5X Denhardt's solution, and 0.1 mg/ml salmon sperm DNA. The hybridisation was carried out at 60 °C for RNA probes and at 42 °C for DNA probes. The filters were washed three times in 5x SSC, 0.1 % SDS and three times in 0.1 x SSC, 0.1 % SDS at 65 °C; 15 min for each washing.

2.6.3 Visualisation of Southern and Northern blots

Following washing, filters were wrapped in cling film and exposed either to X-OMAT photographic film (Kodak) with intensifying screens, or to a Fuji imaging plate Type III-s for imaging and quantification with the Fuji bas 1000 phosphor-imaging system.

2.7 Bombardment of DNA

2.7.1 Bombardment procedure

N. benthamiana seeds were sterilised with 0.25% sodium hypochlorite and were germinated for 7-10 days on MSR6 medium. One day before bombardment the seedlings in groups of 10-12 were transferred onto fresh MSR6 medium distributed over a 3.2 cm^2 target area. DNA coating and particle bombardment were carried out as described previously (Vain, 1993). Each group of 10 seedlings was bombarded twice with 163µl of gold particles coated with 326 ng of DNA and accelerated at 12 Kv. Two weeks after bombardment, seedlings were transferred to a glasshouse between 20°C and 25°C.

2.7.2 Bombarded DNA

The pUC35S-GFP construct (Chapter 5, Figure 5.2) was obtained by inserting the 35S:GFP4 expression cassette from pBIN-35S:GFP4 (Haseloff et al., 1997) as a *Hind*III-*Eco*RI restriction fragment into pUC19 (Yanisch-Perron et al., 1985). The GFP construct was obtained by inserting the full-length GFP open reading frame from pBIN35S:GFP4 (*Bam*HI-*Sac*I restriction fragment) in pUC19. The "G.." fragment (Figure 4) was PCR-amplified from pBIN 35S:GFP5 (Haseloff et al., 1997) using primers GGATCCAAGGAGATATAACAA and AAATCGATTCCCTTAAGCTCG (pos1 and pos453 in the GFP5 cDNA, respectively). The "..P" fragment (Figure 4) was PCR-amplified from pBIN35S:GFP5 using primers AGCTTAAGGGAATCGAT and CTTAGAGTTCGTCATGTTTGT (pos454 and pos813 in the GFP5 cDNA, respectively). The series of PCR-amplified fragments used for the study of the effect of

the length of homology between epiGFP and intGFP (Figure 4D) was obtained from pBluescript in which the complete GFP5 cDNA was inserted as a *Bam*HI-*Sac*I restriction fragment. Primer combinations used for each amplification are:

(AGCTTAAGGGAATCGAT-TTGTGGCCGAGGATGTTT);

(AAATCGATCCCTTAAGCTCG-GGGTAACGCCAGGGTTTTCC);

(AGTAGTGACAAGTGTTGGCC-AGCGGGCGCTAGGGCGCT);

(TGACAGAAAATTTGTGCCCATT-GTAAAGCACTAAATCGGAACC);

(TTGGGACAACTCCAGTGAAAA- CCACTACGTGAACCATCAC).

The ...P and GF. constructs are respectively linear ClaI-SalI and BamHI-ClaI restriction fragments from the GFP construct described above. The pUC35S-GUS construct (Chapter 5) was kindly provided by Isabelle Malcuit.

2.8 Plants and Viruses

All work involving virus infected material was carried out in containment glasshouses under MAFF license PHF 1420C/1773(12/1996).

2.8.1 Transgenic plant material

Independent lines of *Nicotiana benthamiana* plants carrying the GFP5 transgene (Haseloff et al., 1997) were generated by the *A. tumefaciens-mediated* leaf disk transformation method (Horsch et al., 1985). For transformation, we used the disarmed *Agrobacterium* strain GV-3101 containing the binary vector pBIN35S:GFP5 (Haseloff et al., 1997).

2.8.2 Propagation of plants in greenhouse

Nicotiana clevelandii, *Nicotiana benthamiana*, and *Nicotiana tabacum* were germinated on 1:1 mixture of JIC compost and peat, then grown individually in pots at 25 °C during the day and 20 °C at night in a containment glasshouse. Supplementary winter lighting from halogen quartz iodide lamps provided a 16 hour day length.

2.8.3 In vitro propagation

N. benthamiana leaves were harvested from greenhouse-grown plants. Leaf disks were aseptically plated onto MSR6 medium complemented with 1 mg/l 6-Benzylaminopurine and 0.1 mg/l α-Naphthaleneacetic acid.

2.8.4 Wild-type viruses

Isolates of alfalfa mosaic virus (AMV), foxtail mosaic virus (FoMV), narcissus mosaic virus (NMV), nandina virus X (NVX), viola mosaic virus (VMV) and tomato bushy stunt virus (TBSV) were obtained from Roger Hull from the JIC collection. Cowpea mosaic virus (CPMV) was obtained from George Lomonosoff at JIC. African cassava mosaic virus (ACMV) was obtained from John Stanley at JIC (UK). TRV-PPK20 was obtained from John Bol (Leiden University, Netherlands). Other viruses were obtained from a lab collection. These are Tobacco mosaic virus (TMV) strain U1, potato virus X (PVX) strain UK3, potato virus Y (PVY) strain N, cucumber mosaic virus (CMV), tomato black ring virus (TBRV) strain W22 and rice yellow mottle virus (RYMV) strain N.

2.8.5 Recombinant viruses

Cloned copies of wild type PVX (pTXS) as well as the PVX vectors pP2C2S, PVX-GFP and PVX-GUS have been described previously (Baulcombe et al., 1995; Chapman et al., 1992; Kavanagh et al., 1992). PVX-GF was made by replacing the original GFP insert in pPVX204 by the mGFP5 insert from pBin-35S-mGFP5 (Haseloff et al., 1997) and by removing the 354-bp fragment between a ClaI site (position 465 within the GFP5 coding sequence) and a SalI site at the 3' end of GFP5 (position 818). pTXMV-2b contained a 500 bp DNA fragment derived from nucleotides 2410-2908 of CMV RNA 2 (Ding et al., 1995) and thus the complete 2b coding sequence (CMV RNA 2, nucleotides 2410-2712. This inserted CMV sequence was rendered non-coding in pTXYMV-2bΔ by a single nucleotide substitution (T to A) that converted the fourth codon (TTG) of the 2b open reading frame to a stop codon (TAG). The P1 protein sequence of a rice yellow mottle virus isolate from Nigeria (Pinto et al., 1999) was amplified using the following 5' phosphorylated primers; ATG ACT CGG TTG GAA GTT C3' for the intact protein (P1) and ATC ACA CGG TTG TAA GGT TC3' for an untranslatable protein (mP1). The phosphorylated downstream primer used for amplification was CAT CCC GTG TCA GTC TG. The two PCR fragments were cloned into the *Eco*RV site of the p2C2S vector. The orientation of RYMV PCR fragments was confirmed by colony-PCR using antisense primer in the vector sequence at the 3' end of the p2C2S multiple cloning site (GTA GTT GAG GTA GTT GAC CC) and the two sense RYMV 5' primers described above. PVX-AC2 and PVX-mAC2 were provided by John Stanley at JIC (UK). PVX-HS142 and

PVX-HS160 referred to as PVX-19k and PVX-m19k, respectively, were provided by Andrew Jackson.

All the constructs used in chapter 8 were based on pPVX204 which is a PUC19-based vector in which the full length PVX vector is inserted between the 35S promoter and the Nos terminator (Chapman et al.,1992). The entire coat protein ORF was removed from PVX-GFP by digestion with SalI and XhoI and subsequent religation, leading to PVX-GFP-ΔCP. PVX-GFP-ΔTGB-ΔCP was generated by digestion of PVX-GFP-ΔCP with AvrII and EagI, which removed the 3' end of the replicase ORF, the entire TGB and the 3' end of the GFP5 ORF. To restore the replicase and GFP functions, a 3-way ligation was performed with two DNA fragments that had been PCR amplified from PVX-GFP-ΔCP and digested as described bellow. Amplification with
5'-GCACAGATTTTCCTAGGCACGTTATC and
3'-GAAAGAAATTGGgccggctcttgaac (EagI site underlined) led to a DNA fragment corresponding to the 3' end of the replicase ORF that was subsequently digested by AvrII and EagI; amplification with
5'-cagaaaccggccgctagcGGGCCATTGCCG (EagI site underline) and
3'-TGTACTGCTTGAGATTTACAGCT led to a DNA fragment corresponding to the 5' end of GFP5 ORF that was subsequently digested by EagI. PVX-GFP-Δrep-ΔCP and PVX-Δrep-GFP-ΔTGB-ΔCP were generated by digesting PVX-GFP-ΔCP and PVX-GFP-ΔTGB-ΔCP, repectively, with BglII and religation, generating a 1729-nt deletion in the replicase ORF. Individual TGB mutants were generated by introducing previously characterized mutations into the PVX-GFP-ΔCP background. PVX-GFP-Δ12k-ΔCP was

made by inserting an ApaI-BstBI restriction fragment of PVX-GFP-12D into ApaI-BstBI digested PVX-GFP-ΔCP. PVX-GFP-Δ8k-ΔCP was generated by inserting an ApaI-BstBI restriction fragment of pTXS-Δ8K-GFP (kindly provided by Dr Simon Santa Cruz, Scottish Crop Research Institute, Dundee) into ApaI-BstBI digested PVX-GFP-ΔCP. PVX-Δ 8K-GFP has a mutation in the start codon (M→T) of the 8kDa protein that also introduces an in-frame STOP codon without altering the coding capacity of the overlapping 12kDa protein ORF. PVX-GFP-Δ25k-ΔCP was generated from pTXS-GFP-ΔApa/Apa that has a 354-nt deletion in the 25kDa ORF, between an ApaI site inserted by mutation of nucleotides 4588-4591 in the PVX genome and an ApaI site existing naturally at position 4945. The deletion was then introduced as an AvrII-BstBI fragment into AvrII-BstBI digested PVX-GFP. Finally, the PVX-GFP-25k$_{FS}$-ΔCP construct was generated by inserting an AvrII-BstBI restriction fragment from pTXS-GFP3A (kindly provided by Dr Simon Santa Cruz, Scottish Crop Research Institute, Dundee) into AvrII-BstBI digested PVX-GFP-ΔCP. pTXS-GFP3A carries a 4bp deletion, resulting from removal of the 3' overhang (T4 DNA polymerase) of an ApaI digestion at nucleotide 4945 in the PVX genome. This mutation causes a frameshift in the 25kDa ORF starting at amino acid 154 and introduces an in-frame STOP codon at amino acid 159, leading to a truncated protein (C-terminal deletion of 73 amino acids). Constructs carrying fragments of endogenous genes (PDS and Rubisco) were all derivatives of the above vectors. The unique PmlI blunt site in GFP5 was used to clone the corresponding inserts. All the constructs described here were confirmed by sequencing and inserted as SacI fragments into the T-DNA of the pBin19 (Bevan, 1984) or pSLJ (Jones et al., 1992) binary vector plasmids.

2.8.6 Plant inoculation

2.8.6.1 Virus RNA inoculations

0.5 µg of *in vitro* virus transcript RNA in 50 µl SDW was gently rubbed onto 5-10 leaves which had been dusted with carborundum (600 mesh silicon carbide, BDH). For TRV NM inoculum total RNA was extracted from leaves of *N. clevelandii* infected from transcripts, aliquoted in 70% ethanol, and stored at -70°c. This RNA was resuspended in SDW to 0.2 µg/µl and 5 µl was rubbed onto carborundum-dusted leaves. Within 5 min inoculated leaves were washed with water.

2.8.6.2 Sap inoculations.

Infected plant tissue was ground in a pestle and mortar with 50 mM phosphate buffer and sand. The solution was centrifuged for 1 min at 3000 rpm. The supernatant was stored in aliquots at -20°c. For inoculation 10µl of sap was rubbed onto carborundum-dusted leaves. Within 5 min inoculated leaves were washed with water.

2.9 Agrobacterium-mediated transient gene expression and virus inoculation

2.9.1 General method

Agrobacterium-mediated transient gene expression was done as described previously (English et al., 1997) with the following modifications. *A. tumefaciens* was grown to an

O.D.$_{600}$ of 1-1.5 in L broth supplemented with antibiotics and 10 mM MES pH 5.6. The culture was centrifuged and resuspended in 10 mM $MgCl_2$, 10 mM MES, 150 µM acetosyringone and kept at room temperature for 3 hours. The culture was infiltrated into the underside of a leaf using a syringe (without needle), simply by pressing the syringe on the underside of the leaf while exerting a counter-pressure with a finger on the other side. Plants were kept in the greenhouse, and transient gene expression was monitored 2-5 days later. For co-infiltrations, equal volume of both *Agrobacterium* cultures (OD_{600}=1) were mixed before infiltration. For single infiltration, cultures containing the 35S-25k construct were also diluted up to OD_{600}=1 to avoid toxicity to the plant cells.

2.9.2 Binary vector and DNA constructs used for Agrobacterium-mediated transient gene expression

pBin19 and pSLJ have been described previously (Bevan, 1984; Jones et al., 1992). To remove the NPTII cassette from the pBin19 T-DNA (Chapter 4) the pBin19 vector was digested with *Pst*I . The two co-migrating bands corresponding to the vector backbone were excised, electro-eluted and religated, leading to pBin19-noKAN. To construct the epiGFP vector (Chapter 4), the GFP5 cDNA (Haseloff et al., 1997) was amplified by PCR and cloned as a blunt fragment into the *Sma*I site of the expression cassette of pJIT61 (JIC), leading to pJIT61-GFP. The GFP cassette was then excised as a *Kpn*I-*Xho*I restriction fragment and was inserted into *Kpn*I-*Sal*I digested pBin19-noKAN.

The 35S-25k and 35S-25k-ΔATG constructs (Chapter 8) are based on pBin19 engineered with the 35S expression cassette of pJIT61 (JIC), inserted as a *Kpn*I-*Xho*I restriction fragment. The 25 kDa inserts were PCR fragments amplified from pPVX204 (Chapman et al.,1992), using the Pfu polymerase (Promega). For 35S-25k-ΔATG, the start codon was omitted in the forward primer. Both constructs were confirmed by sequencing.

2.9.3 Induction of systemic silencing

Agrobacterium cultures were prepared as described above and brought to saturation. Resuspension was also as described above. Infiltration of the silencing-inducing strain was performed on three weeks old seedlings. For routine induction of systemic silencing, a maximum number of leaves were infiltrated, avoiding cotyledons.

2.10 Visualisation of reporter gene expression

2.10.1 In planta visualisation of the green fluorescent protein

GFP was visualised *in planta* using a 100W long-wave ultra violet lamp (UVP, Upland CA 91786, Black Ray model B 100AP). Photographs were taken on 400 ASA Kodak Ektachrome Panther film through a Wratten 8 filter, with exposure times of 15-90 seconds depending on the distance from the plant.

2.10.2 Microscopy

Close ups were obtained using a LEICA MZFLIII dissecting stereomicroscope coupled to a fluorescence module. The filter set used for GFP imaging was the GFP-plus fluorescence set from Leica (excitation 480nm, dichromatic beam splitters, 505nmLP, Barrier filter 510nmLP). Photographs were produced using LEICAMPS60 device coupled to the stereomicroscope. Confocal microscopy was performed under a Leica DMR module coupled to a Leica TCS-NT system. A 100 mW Argon ion laser was used to produce blue excitation light at 488 nm (emission filter 522 nm).

2.10.3 GUS histochemistry

Histochemical staining of leaf material was performed using 5-bromo-4-chloro-3-indoyl glucuronide (X-gluc) as described previously (Jefferson et al., 1986). Leaves were vacuum infiltrated with a solution containing 0.5 mg/ml X-gluc, 50 mM phosphate buffer pH 7, 0.5% Triton® X-100, 1 mM EDTA, and incubated for 4-16 hours at 37°C before clearing with 70% ethanol.

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CHAPTER 3

Initiation and Maintenance of Virus-Induced-Gene-Silencing in transgenic and non-transgenic plants

3.1 Abstract

A green fluorescent protein (GFP) transgene was silenced in plants infected with potato virus X (PVX) vectors carrying GFP inserts (PVX-GFP) and the phytoene desaturase (PDS) gene of *Nicotiana benthamiana* was silenced in plants infected with PVX-PDS. This virus-induced gene silencing (VIGS) is post-transcriptional and cytoplasmic because it is targeted against exons rather than introns of PDS RNA and against secondary-challenged viral RNAs. Although PDS and GFP RNAs are most likely targeted through the same mechanism, the VIGS phenotypes differed in two respects. PDS mRNA was targeted by VIGS in all green tissue of the PVX-PDS-infected plant whereas PVX-PDS was not affected. In contrast VIGS of GFP was targeted against PVX-GFP. Initially, VIGS of GFP was initiated in all green tissues, as with PDS VIGS. However, after 30 days of infection, GFP VIGS was no longer initiated in newly emerging leaves, although it was maintained in tissue in which it had already been initiated. Based on this combined analysis we propose a model for VIGS in which the initiation of VIGS is dependent on the virus and maintenance is virus independent. A role for VIGS in natural infections and its potential applications as a tool for functional genomics in plants are discussed.

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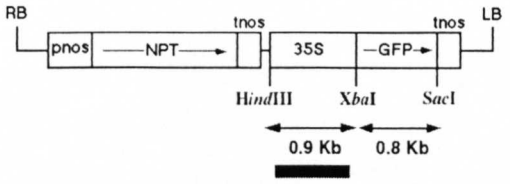
Here I provide an analysis of VIGS that was designed to shed light on the underlying mechanism and the relationship of VIGS, post-transcriptional gene silencing, and anti-viral defense. VIGS targeted against a green fluorescent protein (GFP) transgene was compared to VIGS of the endogenous phytoene desaturase (PDS) gene. Based on the different features of VIGS in these systems, a model is produced, in which the mechanism of VIGS involves separate initiation and maintenance stages. These findings show that VIGS and transgene-mediated gene silencing shares similarities and reinforce the predicted role of gene silencing in natural virus defense.

PAGE NUMBERING AS ORIGINAL

single locus (Figure 3.1-C). A similar conclusion was drawn from hybridisation with a 350bp DNA fragment corresponding to the 3' end of the GFP coding region (data not shown).

A



B

	Theoritical	GFP 16c	GFP 17b	GFP 8	GFP Y
Red	10	13	11	12	9
Green	30	27	29	28	31
Total	40	40	40	40	40

C

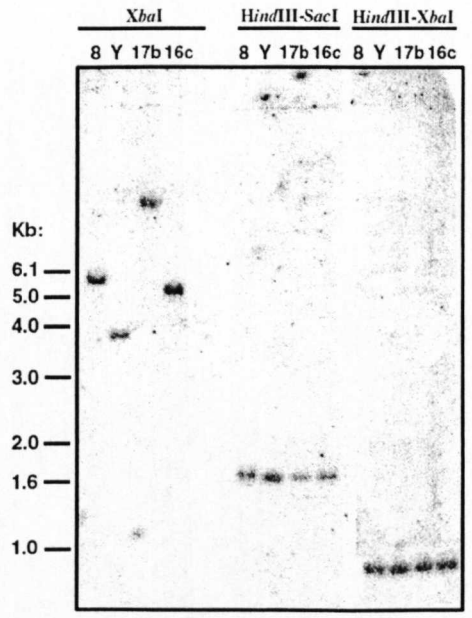


Figure 3.1. Characterisation of the GFP transformants of *N.benthamiana*. (**A**) The 35S-GFP construct was assembled in the pBin19 T-DNA (pnos: *nos* promoter, tnos: *nos* terminator, 35S: CaMV-35S promoter, RB: right border, LB: left border). (**B**) F1 segregation analysis of the 4 transgenic lines, as assessed under UV illumination. The table indicates the number of plants appearing green fluorescent as opposed to red fluorescent. The theoritical ratio for one insertion locus is indicated on the left, alongside the ratios observed for each line (**C**) Southern analysis of total DNA extracted from each of the 4 transgenic lines. The DNA was subjected to 3 different restriction digests and probed with a [^{32}P] dCTP labelled H*ind*III-X*ba*I DNA fragment (shaded box) isolated from the construct depicted in (A). The sizes indicated on the left correspond to the bands of a DNA ladder that was run on one track of the agarose gel and revealed by ethidium staining before blotting.

3.3 VIGS of the GFP transgene as a 3-phases process

To investigate VIGS in those transgenic lines, we constructed the PVX-GF vector in which the 470-nucleotide insert lacked the 3'end of the GFP coding region (Figure 3.2A).

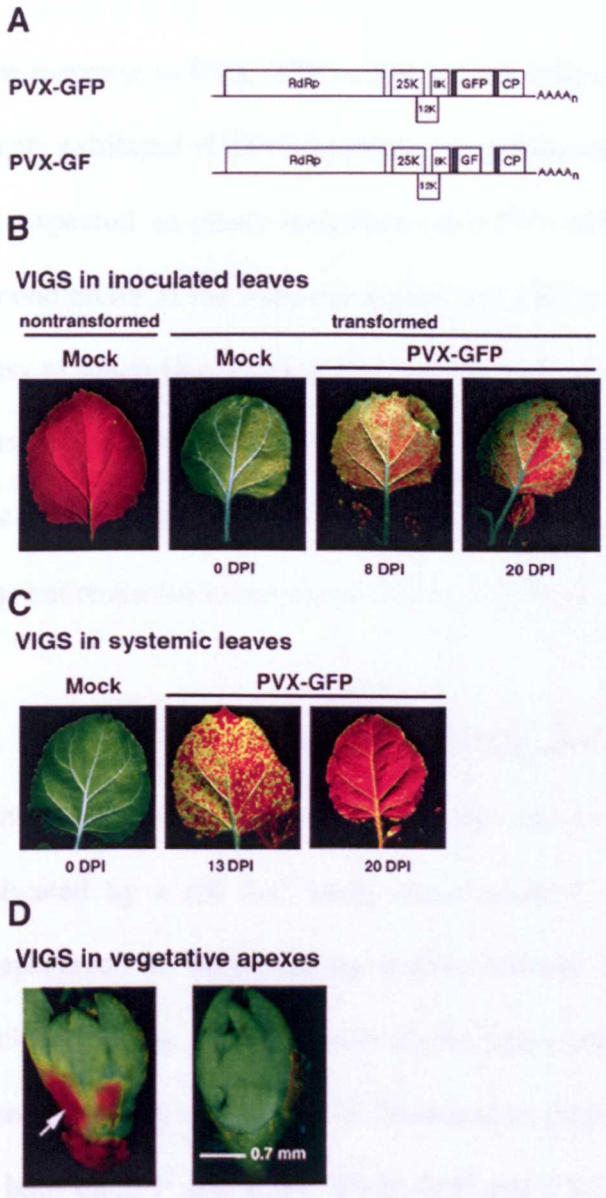


Figure 3.2. VIGS of the GFP. (**A**) The genome organization of PVX vectors used in VIGS of the GFP transgene. The PVX open reading frames are shown as RdRp (RNA-dependent-RNA polymerase), 25K (25K protein), 12K (12K protein), and 8K (8K protein), and the inserts were the GFP open reading frame either intact or with the 3' region deleted. In these vector constructs the viral sequences were coupled to the 35S promoter and the plasmid DNA was inoculated directly to plants. (**B**) The inoculated leaves (PVX-GFP) photographed under UV light at 8 an 20 DPI. (**C**) The uppermost systemic leaves of plants that were either mock inoculated or inoculated with PVX-GFP, as indicated. The leaves were photographed under UV light at 13 and 20 DPI. (**D**) The growing point of GFP transgenic plants photographed under UV light at 20 DPI with a mock inoculum or with PVX-GF. The arrow indicates the regions closest to the apical meristem in which VIGS of the GFP could be observed.

Using the deleted 3' end of the GFP sequence as probe, it was then possible to assay the transgenic GFP RNA independently of the PVX-GF RNA. In some of the experiments

we also used PVX-GFP expressing the full-length GFP RNA. When the same results were obtained with both PVX-GF and PVX-GFP, we refer to these viruses as PVX-GF(P). To avoid confusion we also refer to intGFP from the integrated transgene and vGFP from PVX-GF(P). Although the data presented here are all from one line (GFP8), similar results were obtained with all four lines.

The response to PVX-GFP in these plants followed three phases. Initially, the transgenic plants exhibited vGFP fluorescence superimposed on the fluorescence from the intGFP. As expected, in plants inoculated with PVX-GF there was no vGFP fluorescence. The second phase of the response started at 8 DPI in localized regions of the inoculated leaf. Loss of green fluorescence was manifested as expanding spots that corresponded to the primary sites of initial infection, as observed under normal light (Figure 3.2-B and data not shown). At further time-points, however, loss of GFP was much more extensive and was not restricted to the sites of initial infection.

At 10-15 DPI, the second phase of VIGS was investigated in fully infected, systemic leaves. It was first characterized by the loss of intGFP only (Figure 3.2-C, 13DPI), as indicated by a red leaf background under UV light. At this stage, vGFP was not suppressed, as indicated by green fluorescent spots on the red fluorescent leaf background. By 20 DPI, however, the upper leaves of PVX-GFP-infected plants were homogeneously red under UV illumination (Figure 3.2-C, 20 DPI), indicating silencing of both intGFP and vGFP. PVX-GFP and PVX-GF both silenced intGFP to the same extent and over the same time course. IntGFP silencing was evident in roots, stems, seed capsules, and flowers and was most pronounced in the upper leaves and axillary shoots.

In contrast, as shown in Figure 3.2-D, the floral (data not shown) and vegetative apexes remained green fluorescent indicating either that the virus had not entered these tissues or that they lacked the potential to silence GFP.

In the third phase, beginning at about 28 DPI, VIGS of the GFP was maintained in parts of the plant that had already become silenced. However, there was faint green fluorescence in the tissue emerging from the growing point and by 41 DPI, the newly emerging tissue exhibited full GFP fluorescence. These 3 phases were observed consistently in at least 3 independent experiments involving more than 4 plants each.

3.4 IntGFP RNA levels in infected plants

Northern analysis of intGFP RNA extracted from tissues sampled at each of these 3 phases was consistent with the silencing phenotypes described above. In the first phase, VIGS of intGFP in the PVX-GF-inoculated leaves was confirmed at 10DPI by the decrease of intGFP RNA (revealed with the '..P' probe), when compared to the intGFP RNA levels in similar, mock-inoculated leaves. In the uppermost leaves of the plants, at 13 DPI (second phase), the level of intGFP mRNA in the systemic leaves was below the limit of detection and at least 98% lower than in mock-inoculated plants (Figure 3.3-B, 13dpi). The uppermost leaves also contained low levels of intGFP mRNA at 20 DPI, but at 27 and 34 DPI, corresponding to the third phase of VIGS, the intGFP mRNA in these uppermost leaves was present at detectable levels (Figure 3.3-B, 27 and 34 DPI).

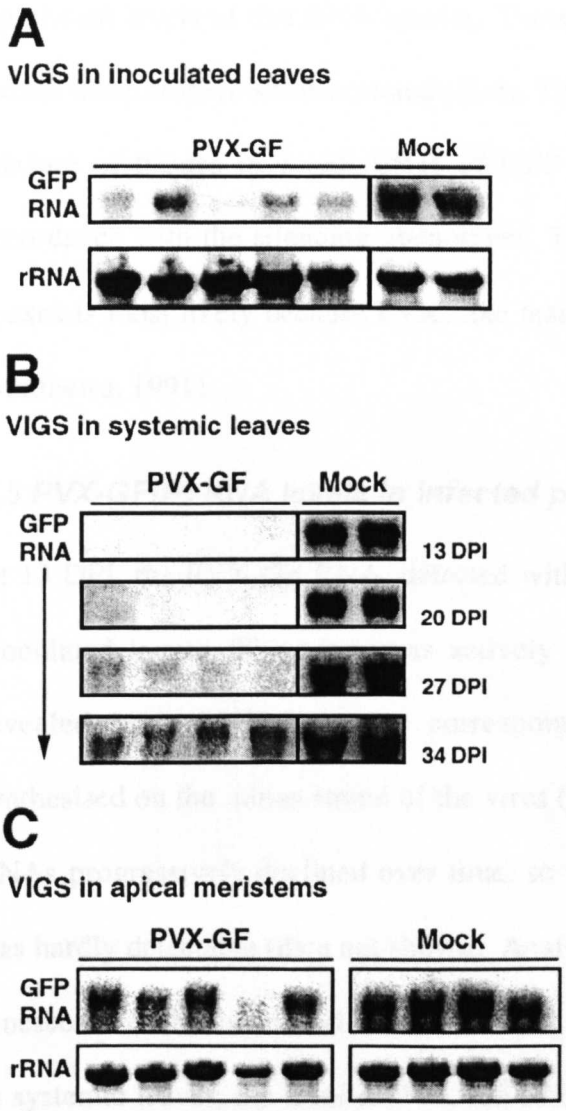


Figure 3.3. intGFP RNA levels. (**A**) intGFP mRNA levels in leaves of of *N. benthamiana* line GFP8 that had been inoculated with mock or with PVX-GF 13 days previously. After RNA extraction, 10 μg of was loaded in each lane. (**B**) intGFP mRNA levels in systemic leaves of mock-inoculated or PVX-GF-inoculated plants of *N. benthamiana* line GFP8. RNA samples were harvested at the indicated DPI from the uppermost systemic leaves of plants and 10 μg was loaded in each lane. (**B**) GFP RNA levels in pooled apical and floral apexes from mock-inoculated and PVX-GF-inoculated plants of line GFP8. RNA samples were harvested at 20 DPI and 10 μg was loaded in each lane. Samples in (**A**) (**B**) and (**C**) were assayed by RNA gel blotting using a phosphorus-32-labeled 3' fragment of the GFP cDNA (...P) as probe. Each lane corresponds to an individual plant. rRNA: ribosomal RNA confirming equal loading.

Analysis of the intGFP RNA in the apexes was performed at 20 DPI (corresponding to the second phase of VIGS) when silencing was extensive in PVX-GF(P)-infected plants. Many floral and vegetative apexes were roughly dissected out from individually silenced plants and total RNA extracted from those pooled tissues was subjected to Northern analysis (Figure 3.3-C). This analysis confirmed that, in contrast to the extreme

suppression of intGFP RNA in expanded leaves (Fig 3.3-B), 20DPI), apexes contained significant levels of this RNA species. These levels were not as high as in control apex tissues taken from mock-inoculated plants. This was probably because the samples were a mixture of tissues in which VIGS of GFP was differentially set. This result was in accordance with the silencing phenotypes. The reason why VIGS was less operative in apexes is most likely because PVX, like many plant viruses, does not access meristems (Matthews, 1991).

3.5 PVX-GF(P) RNA levels in infected plants

At 13 DPI, the PVX-GF RNA, detected with a full-length GFP probe, was high in the inoculated leaves. This virus was actively replicating because the Northern analysis revealed several RNA species corresponding to the subgenomic RNAs that are synthesised on the minus strand of the virus (Figure 3.4-A). However, the levels of these RNAs progressively declined over time, so that by 27 DPI, replicating PVX-GF RNA was hardly detectable (data not shown). Analysis at further time-points was prevented by senescence of the inoculated leaves.

In systemic leaves, the level of PVX-GF RNA also declined during the second phase of VIGS. However, the reduction in viral RNA, as shown in Figures 3.4-B, was slower than the reduction of the intGFP mRNA (Figure 3.3-B). At 13 DPI, when intGFP mRNA was undetectable (Figure 3.3-B), PVX-GF RNA was as abundant in the GFP transgenic plants as it was in the non transgenic plants (Figure 3.4-B). However, by 20 DPI, the PVX-GF in the GFP transgenic line was 95% lower than in non transgenic plants (Figure 3.4-B), 20 DPI). The level further decreased and remained below the level of detection until 41 DPI when the experiment was terminated (Figure 3.4-C). The elimination of viral RNA

corresponded to the third phase of GFP VIGS, as described above, when newly emerging leaves had progressively higher levels of GFP mRNA (Figure 3.3-B). The levels of viral RNA exhibited similar kinetics when the GFP transgenic plants were inoculated with PVX-GFP (data not shown).

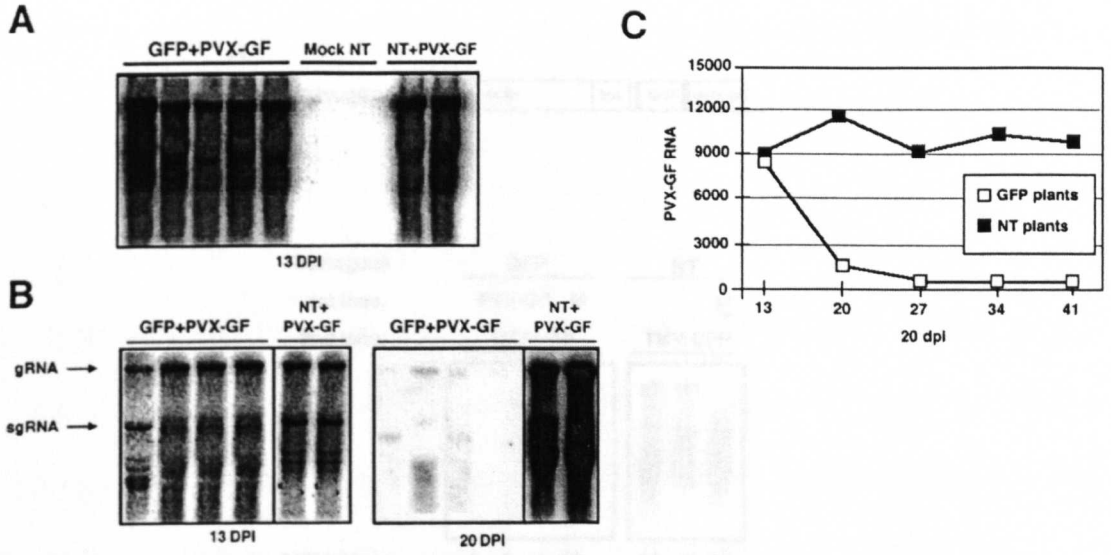


Figure 3.4. vGFP RNA levels. (**A**) vGFP in inoculated leaves. RNA samples were taken at 13 DPI from leaves of GFP8 (GFP) or non transgenic (NT) lines that had been inoculated with PVX-GF. (**B**) vGFP RNA in systemically infected leaves. RNA samples were taken at 13 and 20 DPI from the uppermost systemic leaves of GFP8 (GFP) or non transgenic (NT) lines inoculated with PVX-GF. In both (**A**) and (**B**), equal amounts (10 μg) of each RNA sample were fractionated by agarose gel electrophoresis and a phosphorus-32-labeled RNA probe for GFP was used to detect the recombinant viral RNAs. The genomic (gRNA) and major subgenomic (sgRNA) RNA species are labeled. Each sample was analyzed in replicate and the gel at the bottom was exposed longer than was the gel at the top to allow detection of the residual low levels of PVX-GF RNA in the samples from the GFP transgenic plants. (**C**) vGFP RNA in systemically infected leaves. RNA samples were taken at the indicated DPI from the uppermost systemic leaves of GFP8 (GFP) or nontransgenic (NT) lines inoculated with PVX-GF. Equal amounts (10 μg) of each RNA sample were fractionated by agarose gel electrophoresis and a phosphorus-32-labeled RNA probe for GFP was used to detect the recombinant viral RNAs. The level of vGFP gRNA in each sample was quantified in terms of PSL units using phosphorimaging equipment (see Methods). Each point represents the average value from three RNA samples.

3.5 Maintenance of VIGS

The analysis shown in Figure 3.4 was based on samples taken from the uppermost leaves in which VIGS would have been recently initiated. To investigate the maintenance of VIGS we also analyzed GFP-silenced leaves lower down the plant, which had been

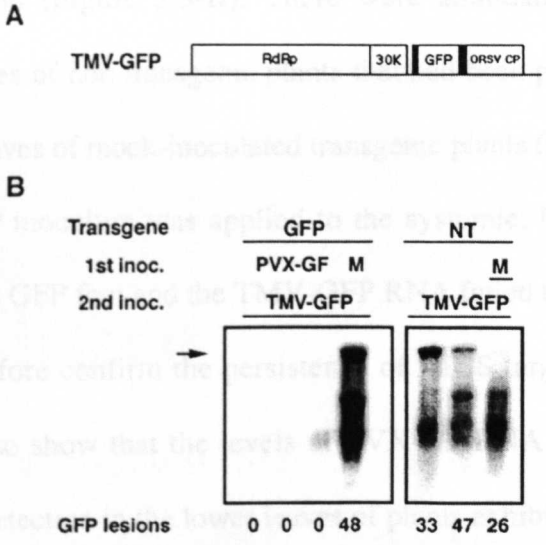


Figure 3.5. Maintenance of VIGS. (**A**) A TMV-GFP vector was used to analyze virus resistance in tissues exhibiting VIGS of the GFP transgene. The TMV open reading frames are shown as RdRp (RNA-dependent RNA polymerase), 30K (30K protein) and odontoglossum ringspot virus (ORSV) coat protein (CP) (Donson et al., 1991). The GFP open reading frame was inserted intact between duplicate CP promoters from the TMV and ORSV genomes (shaded boxes). The vector constructs were assembled as cDNA and transcribed into RNA for inoculation of plants. **(B)** GFP8 or nontransformed plants were initially inoculated (1st inoc.) with PVX-GF or mock inoculated (M). After 21 DPI, the uppermost systemic leaves of these plants were given a second inoculum (2nd inoc.) of TMV-GFP; after another 8 days the GFP infection foci were counted under UV light (GFP lesions). RNA samples (10 μg per lane) were analyzed by gel blotting using a phosphorus-32-labeled probe for GFP. The major genomic (gRNA) of TMV-GFP is indicated.

uniformly silenced at 21 DPI with PVX-GF or PVX-GFP. To determine whether there was persistence of VIGS targeted against intGFP and vGFP RNA, these leaves were

treated with a secondary inoculum of tobacco mosaic virus carrying GFP (TMV-GFP). Persistence of silencing would be manifested as reduced accumulation of TMV-GFP because this secondary-challenged viral RNA would be targeted in a GFP-sequence-specific manner.

The genome organization of TMV-GFP is shown in Figure 3.5-A and the gel blot analysis of TMV-GFP RNA accumulation was carried out on samples taken 8 days post secondary inoculation (Figure 3.5-B). There were abundant TMV-GFP RNAs and infection foci in leaves of non transgenic plants that had been previously inoculated with PVX-GF or in the leaves of mock-inoculated transgenic plants (Figure 3.5-B). In contrast, when the TMV-GFP inoculum was applied to the systemic, GFP-silenced leaves after 21days there were no GFP foci and the TMV-GFP RNA failed to accumulate (Figure 3.5-B). These data therefore confirm the persistence of VIGS targeted against viral RNAs. The gel blot data also show that the levels of PVX-GF RNA and intGFP mRNA were below the limits of detection in the lower leaves of plants exhibiting VIGS of GFP.

The levels of vGFP RNA were further investigated by passage inoculation of sap extracts from the lower leaves of PVX-GFP-inoculated plants exhibiting VIGS of GFP. When inoculated to non transformed plants these extracts failed to produce green fluorescent infection foci, as would be expected if there had been accumulation of PVX-GFP. Also reflecting the absence of PVX-GFP or derivatives, these extracts failed to induce silencing of GFP when inoculated to GFP transgenic *N. benthamiana.* Therefore from these combined back-inoculation data and the gel blot analysis (Figure 3.5-B), we conclude that, in the second phase, VIGS persists in the infected cells in the absence of the inducing virus.

3.6 Comparative analysis of VIGS of the phytoene desaturase endogenous gene

In parallel to the VIGS of the GFP transgene, similar experiments were conducted in wild-type *N.benthamiana* where the phytoene desaturase gene was used as a target of VIGS. These experiments were conducted by M. Teresa Ruiz. In this case, various

Figure 3.6. VIGS of the phytoene desaturase gene (PDS). A wild-type *N.benthamiana* plant exhibit photobleaching associated with VIGS of PDS caused by infection of recombinant PVX in which a fragment of the PDS cDNA has been inserted. The image was taken at 20DPI.

recombinant PVX carrying inserts of the PDS genomic sequence were tested for their ability to cause suppression of carotenoid biosynthesis manifested as photobleaching (Figure 3.6). The outcome of these experiments is outlined below:

❶ VIGS of PDS was first manifested in new emerging leaves, at 10-15 DPI. At first, the bleached regions were confined to the leaf veins. Later, as shown in Figure 3.6 (PVX-PDS), the photobleaching symptoms extended to most of the foliar tissue, although there was always a mosaic of green and white tissue. Stems, axillary shoots, sepals, and seed capsules were all affected.

❷ In contrast to the VIGS of GFP (third phase), VIGS of PDS was consistent over time; tissues emerging as late as 2 months post inoculation continued to show bleaching.

❸ VIGS of PDS was independent of the orientation of a PDS cDNA insert into the PVX

vector. In addition, the potential to cause VIGS was not restricted to a single region in the PDS mRNA sequence.

❹ VIGS of PDS was not observed if the insert corresponded to intron sequences of the PDS gene. The lack of VIGS with the intron vectors suggests that the mechanism is either initiated in the cytoplasm and/or targeted against cytoplasmic RNA and indicates VIGS of PDS is post-transcriptional. This is in agreement with the targeting of the cytoplasmically replicating TMV-GFP and PVX-GFP observed in VIGS of intGFP.

❺ Northern analysis of RNA extracted from photobleached tissues indicated that there was a 85 to 95% reduction of the PDS mRNA levels. However, and in contrast to VIGS of GFP, there was no detectable effect on accumulation of PVX-PDS. The high levels of PVX-PDS were maintained in five independent experiments in tissue that was sampled up to 45 DPI. Moreover, in plants inoculated with sap extracts from both white and green silenced tissue the silencing phenotype developed as quickly and to the same extent as in the transcript-inoculated plants.

3.7 Discussion

3.7.1 Separate stages of VIGS

A key point from our analysis is the separation of initiation and maintenance stages of VIGS. Initiation of VIGS is absolutely dependent on the virus. The target genes were not silenced unless the plants were infected with the corresponding viruses and, if the virus levels declined, as in the third phase, VIGS was not initiated in the newly developing

tissue at the growing point of the plant. However, once VIGS was initiated (as in the leaves exhibiting the third phase), the PVX-GF(P) was no longer required for its maintenance. In the work described here this virus-independent maintenance of VIGS was manifest as the continued absence of GFP and the resistance against TMV-GFP in the leaves from which the PVX-GF(P) had been eliminated.

In previous work with plants carrying viral cDNA transgenes, the "recovery" phenomenon was also a manifestation of virus-independent maintenance of VIGS (Lindbo et al., 1993; Tenllado et al., 1995). Recovery occurred in the upper parts of virus-infected plants provided that there was a high degree of sequence similarity between the virus and the transgene. As in the tissue exhibiting VIGS of GFP here, the recovered tissue was virus free but, nevertheless, showed continued silencing of the viral transgene and remained resistant against subsequent infection by the virus.

It is likely that these distinct initiation and maintenance stages can explain the three phases of GFP VIGS. In the first phase of GFP VIGS, the plants exhibited vGFP fluorescence superimposed on the fluorescence from the intGFP. We envisage that during this first phase of VIGS there would be virus-dependent initiation of VIGS in all of the PVX-GF(P) infected tissue but that the silencing phenotype would not yet be evident. In the second phase of GFP VIGS there would be initiation and maintenance in different parts of the plant. Initiation would be taking place in infected cells at the base of the growing point of the plant and, consistent with this interpretation, we found that phase two plants contain high levels of PVX-GF(P) in the apical zones (data not shown). At the

same time, in the GFP-silenced regions of the plant there would be maintenance of VIGS resulting in progressive loss of vGFP and intGFP fluorescence from leaves and stems that had been previously infected with PVX-GF(P). In the third phase the lower leaves of the plant would continue to exhibit maintenance of GFP VIGS. These leaves remained free of intGFP fluorescence and were resistant against inoculation with GFP virus. However, in the growing point the progressive spread of VIGS through the plant would likely have caused complete elimination of PVX-GF(P). The absence of PVX-GF(P) would mean that VIGS would not be induced in the newly developing leaves and consequently would explain the late increase that we observed in the levels of intGFP RNA and fluorescence. This third phase of VIGS has not been described previously in plants carrying viral cDNA transgenes that were undergoing virus-induced recovery. We consider this difference may be due to the transgene constructs or because the previous experiments were terminated before the third phase had begun (Lindbo et al., 1993; Tenllado et al., 1995)..

3.7.2 Initiation of VIGS

In all examples of virus-induced recovery in transgenic plants carrying viral transgenes, the loss-of-symptom phenotype was observed in the new emerging leaves (Lindbo et al., 1993; Tenllado et al., 1995). To account for this observation, it has been suggested that the absence of symptoms was due to VIGS of the transgene causing virus resistance in the upper parts of the plant. One suggestion was that the silencing was only activated in the upper parts of the plant because viral initiation of silencing only occured in the meristems (Baulcombe, 1996). It was proposed that initiation would proceed during the

transient breakdown of the nuclear membrane in dividing meristematic cells, for instance, *via* RNA-DNA interactions between the cytoplasmic inducer virus and the homologous transgene. However, the data presented here are not consistent with these proposals. First, the analysis of the inoculated leaf clearly indicates that VIGS fully operates in the primarily infected, somatic cells. Second, the study of VIGS in apexes suggests that meristems are precisely the tissues were silencing is not initiated, probably because PVX does not have access to dividing cells. It is likely that in the previous studies on virus-induced recovery in transgenic plants, silencing was also triggered in inoculated and developing leaves that preceded the new growth. Probably, activation of silencing was not recognized in those tissues because, as shown here in the second phase of VIGS of GFP, it was masked by the high levels of symptomatic, recombinant virus.

Initiation of VIGS could be determined by a specific interaction of the viral RNA with the corresponding nuclear gene mRNA. Alternatively, VIGS could be initiated by the virus, independently of the nuclear gene mRNA. Based on the results presented here, neither hypothesis could be ruled out definitively. However, our data are more easily reconciled with the nuclear gene-independent hypothesis because the initiation of VIGS against PDS and GFP was similar. If the nuclear genes were involved it would be expected that initiation of PDS VIGS would be slower or less efficient than GFP VIGS because PDS and GFP are such different genes: PDS is an endogenous gene expressed at a very low level whereas GFP is a transgene expressed at a high level. Furthermore, also consistent with a nuclear gene-independent role of the virus in VIGS, it has been shown that viruses without similarity to a nuclear gene can initiate a gene silencing-like

mechanism (Ratcliff et al., 1997). For these various reasons, we favor the explanation that viral RNA initiates VIGS independently of the corresponding nuclear gene or mRNA.

The proposed role of the virus as an initiator of VIGS, independent of the nuclear gene, could be accommodated in a model of transgene silencing invoking double-stranded (ds) RNA as an initiator of PTGS, based on works carried out in petunia (Metzlaff et al., 1997) and, more recently, in tobacco (Waterhouse et al., 1998) and *Arabidopsis* (Chuang and Meyerowitz, 2000). In petunia, the double strandness was due to secondary structures in the silenced mRNA. In tobacco and *Arabidopsis*, silencing is triggered consistently by inverted-repeat transgene constructs that are engineered to produce dsRNA. In virus-infected cells the dsRNA exists, at least transiently, as a viral replication intermediate (Matthews, 1991). A role for dsRNA in gene silencing could also explain the finding that transgenes encoding replicating PVX RNA are efficient activators of post-transcriptional gene silencing (Angell and Baulcombe, 1997). The fact that initiation of PDS VIGS is independent of the orientation of the insert is also consistent with this proposed involvement of dsRNA as an inducer of PTGS in plants. The subsequent discovery of the phenomenon of RNA interference (RNAi) in animals (approximately three years after the present work was initiated) gave strong support to the notion that dsRNA is a generic and potent activator of PTGS. It was shown in several animal species that production of ds-, as opposed to ss-RNA with homology to nuclear genes results in increased turn-over of the mRNA encoded by the the target gene (Fire et al., 1998). As in PTGS in plants, this effect is sequence-specific and highly efficient.

3.7.3 Maintenance of VIGS

Although a case can be made that initiation of VIGS is independent of the corresponding nuclear gene, the maintenance stage of VIGS is clearly influenced by the nuclear gene. This influence is indicated by the differential features of VIGS caused by PVX-PDS and PVX-GFP. Thus, there was no suppression of PVX-PDS associated with VIGS of PDS, whereas there was suppression of PVX-GFP during the maintenance of VIGS against the GFP. Our interpretation of this difference is that, after the initiation of VIGS of GFP, the GFP transgene produces a factor that has two interrelated activities. One of these activities leads to suppression of PVX-GF(P), whereas the second activity is responsible for maintenance of VIGS in cells from which the PVX-GF(P) has been eliminated. To account for the cytoplasmic effect and the sequence specificity of VIGS, we propose that this factor has GFP RNA as a component.

According to this idea, the PDS endogenous gene would not produce the hypothetical RNA-containing factor that was invoked above to account for VIGS of GFP. Alternatively it could be that the PDS VIGS factor is too rare or lacks an essential feature required for targeting of viral RNA. In this situation, because the hypothetical factor is also required for maintenance of VIGS, the mechanism underlying PDS VIGS would not progress beyond the initiation stage. However, PDS VIGS would persist in the plant due to the continued presence of the PVX-PDS. Alternatively, it could be that the RNA-containing factor is produced in the PVX-PDS-infected cell and that it is able to maintain VIGS of PDS but is unable to effectively target PVX-PDS. In this instance the continuing

high level of PVX-PDS would have masked the involvement of the factor in persistent VIGS of PDS.

What is the feature that influences production of this maintenance RNA-containing factor? Following the present analysis, further experiments were designed to address this question. A similar comparative approach of VIGS of a transgene and VIGS of an endogenous gene was undertaken in *N.benthamiana* (Jones et al., 1999). The transgene was the same GFP transgene as described here. However, the endogenous gene targeted in those new experiments was that encoding the Rubisco small subunit (Rbcs) mRNA, one of the most highly abundant mRNA in plants. VIGS of Rbcs by the PVX vector had similar attributes as the VIGS of PDS described here. It had the same timing, was as extensive and persistent, and was not targeted against the viral recombinant RNA, indicative of a lack of maintenance and the absence of the hypothetical factor (Jones et al., 1999). This result ruled out that the lack of maintenance was a peculiarity of PDS. In addition, because the Rbcs mRNA is several order of magnitude more abundant than the PDS mRNA, this result also ruled out that the level of target mRNA could influence production of the hypothetical maintenance factor. However, analysis of the methylated state of the homologous DNA revealed that the GFP transgene coding region was highly methylated in maintenance-phase tissues, whereas the Rbsc and PDS DNA was not. From these differences, it was proposed that methylation of the GFP transgene could influence the production of the maintenance RNA factor (Jones et al., 1999). For instance, transcription of methylated DNA could lead to synthesis of aberrant RNA molecules that would maintain an active PTGS state in the cell (as postulated in the aberrant RNA

silencing models, see introduction), even after elimination of the inducing virus. The finding that sequence-specific DNA methylation can be induced by a cytoplasmically replicating RNA virus suggests that a species of RNA produced by this virus is able to feedback into the nucleus and mediate the epigenetic switch of PTGS associated with transgenes. Why a transgene as opposed to an endogenous gene, would be prone to this switch remains an open question.

3.7.4 A natural role for VIGS

From the nuclear gene-independent initiation of VIGS proposed in this study, it was predicted that gene silencing would be initiated in cells infected with wild-type PVX as well as with the PVX vector constructs described here. According to this idea, the wild-type virus would accumulate and activate gene silencing that would be targeted against its own RNA. As a result of PVX-targeted gene silencing, PVX replication would slow down. However, in the absence of a homologous nuclear transgene, there would be no RNA-containing factors produced to maintain the gene-silencing mechanism. The suggestion that wild-type viruses elicit gene silencing had also been made by others and was based on the finding that accumulation of PVX and other viruses is higher in cells that are also infected with a potyvirus than in singly infected cells (Pruss et al., 1997). A model was developed in which VIGS is a mechanism that normally restricts virus accumulation in the infected cell and thereby increases accumulation of the other virus in cells that are infected with a potyvirus and a second type of virus. The phenomena in which wild type viruses initiate a gene silencing-like resistance mechanism were also consistent with that suggestion (Covey et al., 1997; Ratcliff et al., 1997).

The subsequent work of Franck Ratcliff gave strong support to the idea that PTGS is a general response to virus infection. It was shown that PVX and tobacco rattle virus (TRV), two unrelated plant viruses, activate an RNA-mediated defense response in wild-type *N.benthamiana* plants. It was further established that this response is functionally equivalent to RNA silencing, thus providing direct evidence that PTGS of nuclear genes observed in VIGS is a manifestation of a natural defense mechanism targeted against a wide range of viruses (Ratcliff et al., 1999).

3.7.5 VIGS as a tool

The combined analysis described in this chapter also revealed the huge potential of VIGS as a technology. The simplest application of VIGS is as a tool in reverse genetics analysis of gene function (Baulcombe, 1999). It is now possible to silence a gene by VIGS and thereby determine the role of the gene product in a few weeks, much quicker than by conventional transgenic antisense or sense suppression technologies. This approach is particularly suited to essential genes that would have lethal phenotypes in mutant or transgenic plants. It is also possible to use cDNA libraries in a forward genetics approach based on VIGS. This strategy has been successfully developed in the lab where VIGS mediated by PVX has been applied on a large scale to identify genes that are required for transduction of pathogen resistance pathways. More recently, a VIGS vector based on TRV, which infects meristems, was developed to circumvent the incapacity of PVX to produce silencing in apexes (Ratcliff et al., 2001). For instance, the use of TRV now allows silencing of homeotic genes. Another interesting aspect of TRV is that it

infects *Arabidopsis*. Efforts are currently focussed on the development of a TRV-based VIGS technology in this model species.

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CHAPTER 4

A systemic, sequence-specific signal of gene silencing in transgenic plants

4.1 Abstract

Various factors including ectopic DNA interactions DNA methylation, transgene expression level and double stranded RNA have been proposed as initiators of gene silencing. However, it has not been possible to investigate these factors systematically in transgenic plants because there are other complex, poorly understood influences on expression of chromosomal transgenes. Here we describe a novel approach to analysis of gene silencing based on *Agrobacterium*-mediated transient gene expression. This approach has revealed the existence of a previously unknown signalling mechanism in plants that mediates systemic gene silencing. The signal of silencing is gene-specific and likely to be a nucleic acid that moves between cells.

4.2 Introduction

The previous chapter described how infection of PVX-GFP consistently activates PTGS of a GFP transgene in *N.benthamiana*. In this system, PTGS was as efficient and as extensive in all of the 4 characterised transgenic lines, indicating that VIGS is independent on the chromosomal position of the transgene locus carried by each line. As explained, it is likely that the consistency and nuclear gene-independence of this form of silencing is due to the fact that VIGS is a mechanism that normally restricts PVX accumulation in the infected cells. Most likely PVX, as part of its replication strategy, produces some RNA species that are potent inducers of PTGS in plant cells. It is possible that the dsRNA replication intermediates of PVX represent some of these triggering RNA molecules.

In contrast to VIGS, PTGS induced by transgene constructs is often inconsistent and unpredictable. PTGS can be activated by single copy, sense transgenes, indicating that factors other than dsRNA may act as trigger of PTGS in plants (Elmayan and Vaucheret, 1996). These factors, however, are difficult to investigate systematically through production of transgenic plants, because there are complex and poorly understood effects exerted by flanking chromosomal DNA on transgene expression (Iglesias et al., 1997). Therefore, because transgene DNA insertion cannot be controlled, no two transgenic lines can be considered similar. In addition to these position effects, analyses are complicated by the fact that transgene loci are often recombined during chromosomal integration. This phenomenon occurs randomly and can result in formation of complex multimeric forms

that are potentially associated with variable amounts of DNA from the transformation vector (Iglesias et al., 1997). These cumulated caveats mean that circumstantial rather than comprehensive studies of the factors influencing transgene-induced PTGS have been carried out so far.

The first step towards understanding of these factors was thus to design an experimental system that reproducibly triggers transgene silencing, without relying on chromosomal integration. Once set up, this system would allow modification of the triggering construct and precise dissection of the features required for induction of PTGS. We describe here the development of such a system, based on *Agrobacteium*-mediated transient expression. This procedure allows 100% activation of transgene silencing in leaves of transgenic plants that are initially non-silenced. In the process of developing this tool, we discovered that transgene-induced silencing is a non-cell autonomous phenomenon mediated by a sequence-specific, systemic signal.

4.3 Agrobacterium-mediated transient expression as a tool to study transgene-induced silencing GFP

4.3.1 Binary vector constructs

We reasoned that transient, as opposed to transgenic expression of silencing-triggering constructs could provide a means to achieve our goal. From previous work in the lab and elsewhere, it was already established that leaf infiltration with cultures of recombinant *Agrobacterium tumefaciens* was an effective method to transfer transgene constructs into

many plant cells of the infiltrated area. We thus applied this technique, using *Agrobacterium* strains carrying various binary Ti plasmid, including one with a GFP reporter gene. The initial target of gene silencing in these experiments was the stably-

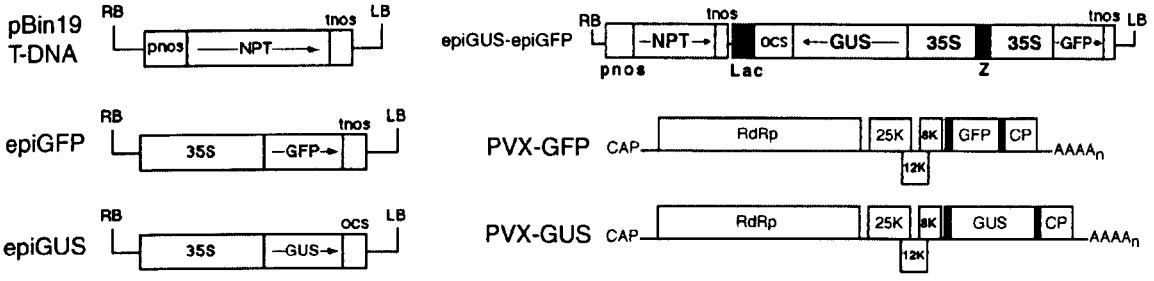

Figure 4.1. Transgene and Viral Constructs used in this study. The NPTII gene in the T-DNA of pBin19 was deleted and used to clone the GFP and GUS expression cassettes, giving rise to epiGFP and epiGUS, respectively. The epiGUS-epiGFP construct was assembled in the non-modified pBin19 T-DNA. A LacZ polylinker was inserted for cloning facilities (pnos: *nos* promoter, tnos: *nos* terminator, 35S: CaMV-35S promoter, OCS: octopine synthase terminator, RB: right border, LB: left border). Structures of PVX-GUS and PVX-GFP are presented (see also Figure 3.2, chapter 3). Expression of the inserted marker genes is controlled by a duplicated coat protein (CP) promoter (shaded boxes).

integrated GFP transgene carried by *Nicotiana benthamiana* line GFP8. We refer to the stably integrated and transiently expressed GFP transgenes as intGFP and epiGFP, respectively. As indicated in Figure 4.1 the epiGFP transgene construct contained the same cassette used to engineer the transgenic GFP *N.benthamiana* lines (Figure 3.1, Chapter 3). We anticipated that upon infiltration, transfer of the epiGFP construct would possibly trigger PTGS of the intGFP. Silencing would be easily monitored under UV illumination, as it would be manifested as loss of green fluorescence in the *Agrobacterium*-infiltrated area, which would thus turn red.

The controls in those experiments were *Agrobacterium* strains that carried either an empty T-DNA (strain epi-0) or a T-DNA with a unique insert of the GUS open reading frame under the control of the CaMV 35S promoter and octopine synthase (ocs) terminator (Figure 3.1). This strain is referred to here as epiGUS. Finally, as a control for

T-DNA transfer, we used the epi-GUS-epi-GFP construct (Figure 3.1), which is a composite of the two expression cassettes carried separately by the epiGFP and epiGUS constructs. The epiGFP and epiGUS constructs were inserted into the T-DNA of the pBin19 binary vector, in which the NPTII gene for plant selection had been removed (Figure 3.1). The epi-0 strain was also based on the NPTII-deleted pbin19 (Figure 3.1). The epiGUS-epiGFP construct was inserted into the intact T-DNA of pBin19. All these constructs were transformed and propagated into *Agrobacterium* GV3101 cells.

4.3.1 Localised activation of silencing is sequence-specific

At 2 days post-infiltration with the epiGUS-epiGFP strain of *Agrobacterium* ,there was expression of both the GUS and the epiGFP reporter genes in the infiltrated tissues of non-transgenic and intGFP transgenic line (Figure 4.2-A, 4.2-B), confirming that T-DNA transfer had occured. In the intGFP transgenic line (Figure 4.2-B) the strong green fluorescence due to the epiGFP was superimposed over a weaker background fluorescence from the intGFP. However, at the edge of the infiltrated zone there was a thin line of red fluorescent tissue (Figure 4.2-B, arrow) indicating that intGFP expression had been suppressed. By 10 days, the infiltrated area had gradually lost green fluorescence from both the epiGFP and intGFP, so that the tissue appeared uniformly red (Figure 4.2-C). A similar response was also observed upon infiltration with the epiGFP strain, in which the T-DNA does not have the GUS expression cassette or the NPTII gene. Northern analysis of RNA extracted from tissues of intGFP plants that had been infiltrated with the epiGUS-epiGFP or epiGFP strain revealed that, at 6 days post-inoculation, the level of both epiGFP or intGFP mRNA was below the detection limit, confirming that gene silencing had been activated (Figure 4.2-D). Activation of silencing was observed in 100% of more than 100 leaves from independent plants.

This effect was not an artefact of the infiltration procedure because similar leaves treated with the epi-0 strain or with water remained green fluorescent. We could also rule out

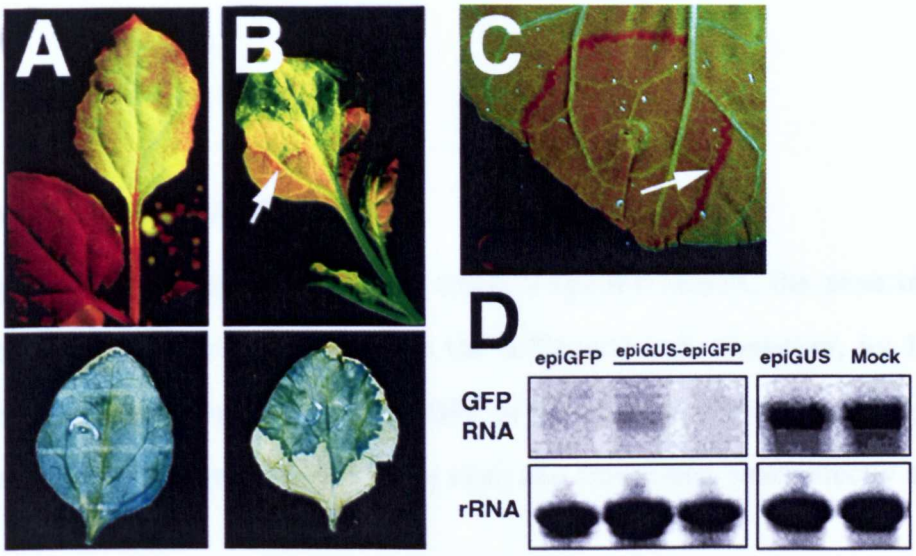

Figure 4.2. Localised activation of silencing of the GFP transgene. (**A-B**) transient expression of GUS and GFP reporter genes in *N. benthamiana*. These images were produced under UV illumination except for the bottom panels that show leaves stained for GUS activity. Expression of epiGFP (top panel) and GUS (bottom panel) in leaves of an non transgenic plant (A) or an intGFP plant (B) that had been infiltrated with the epiGUS-epiGFP strain of *A. tumefaciens* 2 days previously. The arrow in (B) indicates the zone of intGFP suppression at the edge of the infiltrated zone where a line of red fluorescent tissue is observed. (**C**) A leaf of a intGFP plant that had been infiltrated 10 days previously with the epiGFP strain of *A. tumefaciens*. The infiltrated area is dark red, indicating silencing of both the epiGFP and intGFP. Note that the red line is still visible (arrow). (**D**) intGFP and epiGFP mRNA levels in leaves of of *N. benthamiana* line GFP8 that had been infiltrated with various strain of *A. tumefaciens* 10 days previously. After RNA extraction, 10 μg of was loaded in each lane. Samples were assayed by RNA gel blotting using a phosphorus-32-labeled GFP cDNA as probe. The lower panel shows probing of the northern blot with a ribosomal RNA (rRNA) probe to confirm equal loadings of RNA.

that the 35S promoter component of the epiGFP and epiGUS-epiGFP constructs was involved, because there was no suppression of intGFP following infiltration with the epiGUS strain in which the transgene construct contains a 35S promoter (Figure 4.1). Therefore, we conclude from these combined results that suppression of intGFP was a

sequence-specific effect based on the common presence of GFP coding sequences in the intGFP and epiGFP constructs.

4.4 Systemic silencing of GFP

4.4.1 Phenotype of systemic silencing

In the experiments with the epiGFP and epiGUS-epiGFP strains, the zone of intGFP suppression did not spread further within the infiltrated leaf. However, by 18d post-infiltration, we observed suppression of intGFP in the upper, systemic leaves (Figure 4.3-A). This effect was most pronounced in the stem and leaves that were directly above the

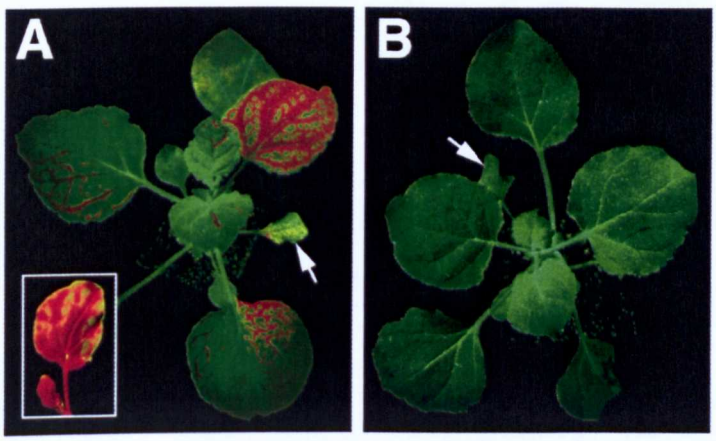

Figure 4.3. Systemic silencing in intGFP plants. (**A**) A plant that had been infiltrated with the epiGUS-epiGFP strain of *A.tumefaciens* 18 days previously. Silencing is very pronounced in axillary shoots (inset). (**B**) A control plant infiltrated with water. The arrows in (A) and (B) indicate the infiltrated leaf.

infiltrated leaf and in the tissues surrounding the veins (Figure 4.3-A). In leaves of the axillary shoots (Figure 4.3-A inset) and in some uppermost leaves (Figure4.3.A) there was complete suppression of green fluorescence due to intGFP. The time-course of intGFP suppression and its pattern of spread through the vegetative parts of the infiltrated plants were consistently observed in more than 10 independent experiments involving 20 plants. Importantly, and as observed for the infiltrated leaf, there was no systemic suppression of intGFP when the plants were infiltrated with the epiGUS or epi-0 strains

of *A. tumefaciens* or with water (Figure 4.3-B). This phenomenon was locus-independent because it was also observed in *N.benthamiana* lines GFP16c, GFPY and GFP17b described in chapter 3.

4.4.2 Systemic silencing confers sequence-specific resistance against homologous viral RNA

In the tissues exhibiting the systemic suppression of intGFP, the steady state levels of intGFP RNA were reduced below the level of Northern blot detection (Figure 4.4-A, lanes 3, 4) indicative of gene silencing. To investigate whether the mechanism of intGFP silencing is transcriptional or post-transcriptional, we exploited previous demonstrations

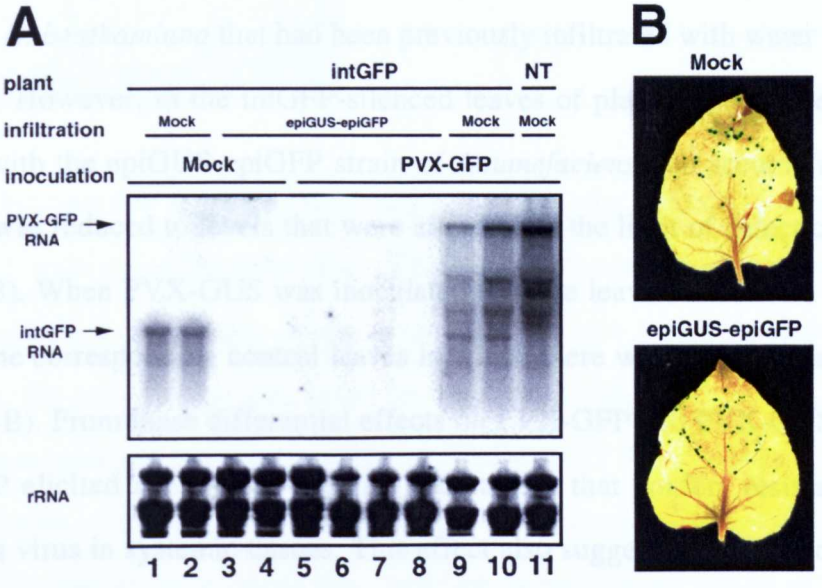

Figure 4.4. Systemic silencing is post-transcriptional. (**A**) Northern analysis of intGFP and PVX-GFP RNA. intGFP plants (GFP) or non-transgenict plants (NT) were infiltrated with either water (Mock), or the epiGUS-epiGFP strain of *A. tumefaciens*. After 20 d, two upper leaves were inoculated with water (Mock) or PVX-GFP. 5 days after virus inoculation, total RNA was extracted from one of the two inoculated leaves and northern analysis on 10μg of RNA was carried out to detect accumulation of the intGFP RNA and PVX-GFP RNA (indicated on the left side of the upper panel). The heterodisperse RNA species in tracks 9-11 represent sub-genomic and degraded RNA species and are typical of PVX RNA samples of inoculated leaves. The lower panel shows probing of the northern blot with a ribosomal RNA (rRNA) probe to confirm equal loadings of RNA. (B) PVX-GUS foci observed on a systemic leaf of an intGFP plant infiltrated with either water (upper image) or with the epiGUS-epiGFP strain of *A. tumefaciens* (lower image). Leaves were inoculated with PVX-GUS and collected after 5 days for GUS staining. When leaves were collected later than 5 days post-inoculation, the GUS foci had spread to the veins, indicating a potential for systemic spread of PVX-GUS independently of intGFP silencing.

that post-transcriptionally silenced transgenes (as assessed by run-on assays) confer resistance against modified potato virus X (PVX) constructs in which there is sequence similarity to the silencer transgene (English et al., 1996). A transgene exhibiting transcriptional gene silencing did not affect the corresponding viral construct. The modified PVX in the present analyses (Figure 4.1) carried either a GFP or a GUS reporter gene. (PVX-GFP and PVX-GUS respectively). The viral inocula were applied to the upper leaves of *N. benthamiana* at 18d post-infiltration with either water or with the epiGUS-epiGFP culture of *A.tumefaciens*. Northern analysis (Figure 4.4-A) revealed that at 5d post-inoculation there was abundant PVX-GFP RNA in leaves of non-transgenic and intGFP *N. benthamiana* that had been previously infiltrated with water (Figure 4.4-A, lanes 9-11). However, in the intGFP-silenced leaves of plants that had been previously infiltrated with the epiGUS-epiGFP strain of *A.tumefaciens*, the accumulation of PVX-GFP RNA was reduced to levels that were at or below the limit of detection (Figure 4.4-A, lanes 5-8). When PVX-GUS was inoculated to these leaves there were as many GUS foci as on the corresponding control leaves in which there was no suppression of intGFP (Figure 4.4-B). From these differential effects on PVX-GFP and PVX-GUS we conclude that epiGFP elicited a sequence-specific mechanism that confers resistance against a homologous virus in systemic tissues. This effect also suggests but does not demonstrate that the intGFP mRNA is silenced at the post-transcriptional level in those tissues.

4.4.3 Systemic silencing is mediated by a signal

We can rule out that the systemic suppression of intGFP was associated with systemic spread of the epiGUS-epiGFP strain of *A.tumefaciens* because there was no detectable expression of GUS in tissues that exhibited systemic suppression of intGFP (Fig. 2h-j). Furthermore, using a selective enrichment procedure, we could not detect *A.tumefaciens* in sap extracts of tissue showing suppression of intGFP. In ten samples the selective

enrichment procedure detected *A.tumefaciens* in 10^{-12}-fold dilutions of infiltrated leaf extracts. However, in forty-five samples from systemic tissue (including stems and

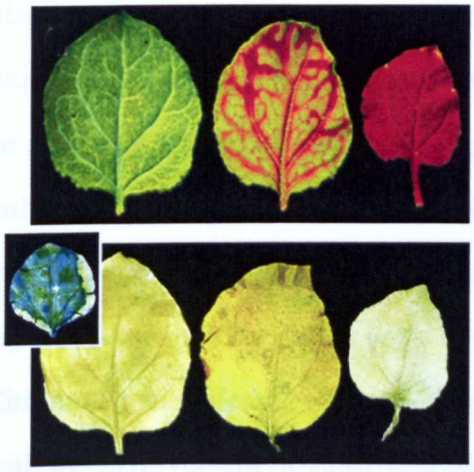

Figure 4.5. UV illumination of upper leaves emerging from the main stem of a intGFP plant infiltrated 18 days previously with water (left), or with the epiGUS-epiGFP strain of *A. tumefaciens*. (middle and right). Leaves were stained for GUS activity (lower panel). The inset shows a leaf infiltrated with the epiGUS-epiGFP strain of *A. tumefaciens* as an internal control for the histochemical GUS staining shown in this figure.

apexes) exhibiting full or partial silencing of intGFP, the infiltrated *A.tumefaciens* was not detected, even in undiluted samples. These sensitive assay methods therefore confirm that *A.tumefaciens* cells were absent from the systemic tissue in which intGFP was suppressed. We can also rule out, based on negative results of a PCR test for GUS DNA, that there was systemic movement of the epiGUS-epiGFP binary vector independently of its *A.tumefaciens* host (data not shown). From these results, we conclude that systemic silencing is mediated by a signal and, from the sequence-specificity of this signal, we infer that it is or at least contains a nucleic acid.

4.5 Discussion

We have demonstrated here that *Agrobacterium*-mediated transient expression of homologous transgene constructs is a rapid and consistent method to trigger transgene-induced PTGS of a stably integrated, highly expressed GFP transgene in *N.benthamiana*. The development of this tool now allows a systematic analysis of the features that are required in the introduced transgene to activate PTGS (see chapter 5). Because this

system is induced, it also allows a direct comparison between silenced and unsilenced state in cells with identical genetic background. A surprising and fascinating outcome of this study is the discovery that silencing can spread systemically through the transgenic plants. A systemic, sequence-specific signal of gene silencing is a novel concept in plant biology. However, it is consistent with data from other experimental systems and could be a general feature of gene silencing in plants. Transgenic petunia exhibiting transgene-induced silencing of genes required for flower pigment biosynthesis exhibit unusual and irregular patterns of pigmentation that could be more easily explained by an extracellular signal rather than by cell lineage-dependent cues of gene silencing (Jorgensen, 1995). Chitinase gene silencing in non-clonal sectors of transgenic tobacco is also consistent with the involvement of an extracellular signal (Kunz et al., 1996). Non cell-autonomous silencing had also been invoked to explain the pattern of transgene-induced silencing of the nitrate reductase gene in tobacco (Palauqui et al., 1996). In parallel to this study, it was shown that silencing of the nitrate reductase and glucuronidase gene is graft-transmitted with 100% efficiency from silenced stocks to non-silenced scions expressing the corresponding target transgene (Palauqui et al., 1997). Transmission was found to be transgene specific and locus independent, as described here for systemic silencing of intGFP. This study also showed that spread of the signal required the presence of a transcriptionally active transgene in the target scions, suggesting that an element of signal propagation required the presence of target transcripts, as opposed to target DNA. This work also confirmed that sequence-specific, systemic silencing was not a peculiarity of the GFP model system.

What is the systemic signal of gene silencing? It is apparently produced when epiGFP is transferred to the cells of the intGFP plant and requires sequence similarity in the coding regions of epiGFP and intGFP. These findings suggest that the GFP or the corresponding DNA or RNA is a component of the signal. Of these, GFP is the least plausible candidate

because there is no mechanism known to us that explains how it could move systemically and specifically target the RNAs of intGFP and PVX.GFP. However, a nucleic acid-based signal could mediate sequence-specific gene silencing via a base-paired or triple helical structure with the target RNA. Moreover, a nucleic acid could move in the plant, perhaps using the channels involved in virus movement. The systemic spread of intGFP silencing (Figure 4.3) is consistent with this suggestion because it apparently follows a course that is similar to the pattern of virus spread in an infected plant (Roberts et al., 1997).

This discovery of a silencing signal also raises a number of additional fundamental questions. How is the signal generated in the infiltrated cells, and how is systemic silencing maintained throughout the plant? What are the plant channels used for signal movement? Is there a natural role for this signalling system? These issues are discussed in the following chapters of this thesis.

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CHAPTER 5

Further characterisation of systemic silencing in transgenic *N.benthamiana*

5.1 Abstract

In this chapter, systemic, post-transcriptional silencing of a GFP transgene is further characterised. It is shown that initiation of signalling by *Agrobacterium*-mediated transient expression is highly dependent on T-DNA transfer efficiency. Bombardment experiments indicate that systemic silencing can be initiated in a few cells and that the presence of a promoter in the introduced GFP sequence is not a prerequisite for initiation. However, there is an influence exerted by the length of homology between the trigger sequence and the target GFP transgene. Surprisingly, the regions of the transgene RNA that are targeted in systemic tissues expand beyond the sequence of the bombarded DNA molecule. Following the initiation step, the sequence-specific signal of gene silencing moves from cells which had received the ectopic DNA, *via* plasmodesmata and phloem channels. Reception of the signal in systemic tissue activates a post-transcriptional, sequence-specific sense suppression mechanism similar to the example of PTGS in transgenic plants carrying sense transgenes. Movement of the signal occurs as a relay process whereby the signal molecule is amplified as it moves away from its site of initiation. Based on these collective observations, a model for systemic silencing is presented, in which initiation and maintenance of the signal requires epigenetic

modifications of transgene DNA. The possible role(s) of systemic silencing in non-transgenic plants are also discussed.

5.2 Introduction

To reduce variations associated with the integration of silencer transgenes in transgenic plants, a transient *Agrobacterium* infiltration assay was devised to deliver the silencer DNA into plant cells. Using this system, it was shown that localised delivery of DNA containing coding regions of a jellyfish green fluorescent protein (GFP) gene activated silencing of a stably integrated GFP transgene, including in tissues that were remote from the infiltrated area. This indicated that GFP silencing was mediated by a signal that moves systemically through the plant. The signal differs from hormones and other previously described signals in plants in that it is sequence-specific. To explain this finding, we proposed that the signal either is, or at least contains, a nucleic acid. Here, I describe how systemic PTGS can be induced following localised delivery of DNA, either by *Agrobacterium* infiltration or biolistically. Bombardement experiments showed that systemic gene silencing in the whole plant can be initiated from a small group of cells. Long distance movement of the signal occurs through the phloem and cell to cell movement is through plasmodesmata. The propagation of the signal involves a long distance relay process in which signal production is amplified as it moves away from the original site of DNA delivery. These findings characterise a signalling system in plants that may have a role in defence and developmental regulation.

5.3 Initiation of systemic silencing

5.3.1 The infuence of T-DNA transfer and binary plasmid on Agrobacterium-mediated initiation of systemic silencing

In the experiments described in this section, systemic silencing was initiated with the epiGUS-epiGFP strain of *Agrobacterium* (Figure 5.1). The T-DNA of the epiGUS-

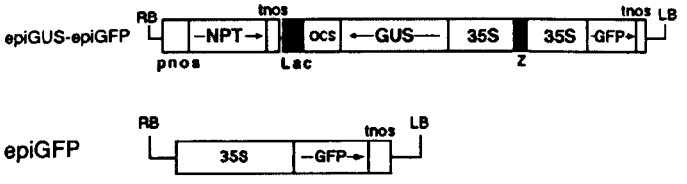

Figure 5.1. Transgene constructs used for *Agrobacterium*-mediated induction of systemic silencing of intGFP.

epiGFP strain would have been transferred into the plant cells in a process that requires expression of the bacterial virulence (Vir) genes (Zupan and Zambryski, 1997). To determine whether systemic silencing is influenced by the efficiency of epiGFP transfer into plant cells, the previously described experiments were repeated under conditions in which the *A. tumefaciens* Vir gene activity was either up- or down-regulated. To down-regulate the Vir genes the *A. tumefaciens* culture was incubated prior to inoculation in the absence of acetosyringone, which is an inducer of Vir genes.

Binary vector	Construct	Aceto	Hypervirulent *Agrobacterium*	No. of plants	No. of silenced plants by 7dpi	No. of silenced plants by 20dpi
PBin19	epiGUS-epiGFP	+	+	30	26	30
PBin19	epiGUS-epiGFP	+	-	100	0	100
PBin19	epiGUS-epiGFP	-	-	30	0	0
PBin19	epiGFP	+	+	30	25	30
pSLJ	epiGFP	+	+	30	0	0

Table 1. Influence of T-DNA transfer and binary vector backbone on systemic silencing induction. The values presented were collected in 3 independent experiments involving at least 10 plants each. dpi: days-post-infiltration; Aceto: culture treated with 100 mM acetosyringone. A plant was considered as silenced if there was loss of GFP fluorescence surrounding the veins of systemic leaves.

Up-regulation of Vir genes was achieved by use of a hypervirulent strain of *A. tumefaciens* (cor308) carrying duplicate copies of VirG, VirE1 and VirE2 (Hamilton et al., 1996). VirG is the transcription activator of all Vir functions; VirE1 and VirE2 are involved in T-DNA transfer and stabilisation in the plant cell cytoplasm and in T-DNA nuclear targeting (VirE2) (Zupan and Zambryski, 1997). From both approaches the results indicated that systemic silencing requires Vir gene function. Thus, with epiGUS-epiGFP cultures produced in the absence of acetosyringone, the onset of systemic silencing was inconsistent from plant to plant and was much slower (40 days post infiltration) than with the cultures prepared in the presence of acetosyringone (around 20 days post infiltration, Table I). Furthermore, the systemic silencing from cultures produced without acetosyringone was restricted to small discrete zones in the upper parts of the infiltrated plants and was much less extensive than in plants infiltrated with acetosyringone treated cultures. Conversely, the use of a hypervirulent *A. tumefaciens* (cor308) host of the epiGUS-epiGFP construct accelerated the development of systemic silencing by several days. Systemic silencing initiated with this strain started at 7 days post-infiltration and was complete by 10 days. Similar results were obtained when the epiGFP construct (Figure 5.1) was used instead of epiGUS-epiGFP (data not shown).

The epiGUS-epiGFP and epiGFP constructs described above (Figure 5.1) were assembled in a pBin19 vector plasmid. However, *Agrobacterium* strains carrying the same epiGFP construct in a pSLJ background (Jones et al., 1992) rather than pBin19 were not able to induce systemic silencing (Table I). The pSLJ plasmids accumulate in *Agrobacterium* at lower copy number than the pBin19 vectors (Jones et al., 1992), and it

is likely that this feature reduces the efficiency of T-DNA transfer from the bacterium to the plant cell. Consistent with this hypothesis, transient expression of the epiGFP construct from a pSLJ background leads to very weak green fluorescence in leaves (data not shown).

From the experiments described above, it was decided that the combination: [pBin19/acetosyringone-treated *Agrobacterium* cor308] would be used in further analysis of systemic silencing triggered by *Agrobacterium*-mediated transfer.

5.3.2 Biolistic activation of systemic silencing

In the experiments described above, epiGFP was delivered by infiltration of *A. tumefaciens* into leaves of intGFP transgenic plants. To evaluate an alternative means of epiGFP delivery, we bombarded small seedlings (5-7 mm long) with gold particles coated with the pUC 35S-GFP plasmid (Figure 5.2-A). This plasmid is based on pUC19 and has the complete 35S-GFP cassette from the epiGFP construct (Figure 5.1). Three weeks after bombardment, 75% of the plants showed systemic silencing of intGFP (Figure 5.2-B, C). As in the *Agrobacterium*-infiltrated plants (Chapter 4, Figure 4.3-A), there was systemic silencing of intGFP throughout the plant except in the growing points of the shoots and roots. This result was consistent and reproducible in seven independent experiments, involving a total of 70 plants (Figure 5.2-A). Systemic silencing of intGFP was never observed when intGFP plants were bombarded with uncoated gold particles or plasmid that did not carry the GFP ORF (data not shown).

In order to estimate the number of cells that receive the delivered DNA, we also bombarded seedlings with a pUC 35S-GUS plasmid and stained the whole plants for GUS activity three days later. We found that, on average, less than 8 randomly distributed individual cells exhibited blue staining in whole seedlings (data not shown). These results indicate that systemic silencing does not depend on the delivery method of epiGFP and, more importantly, that very localised events can initiate production and spread of the sequence-specific signal of gene silencing.

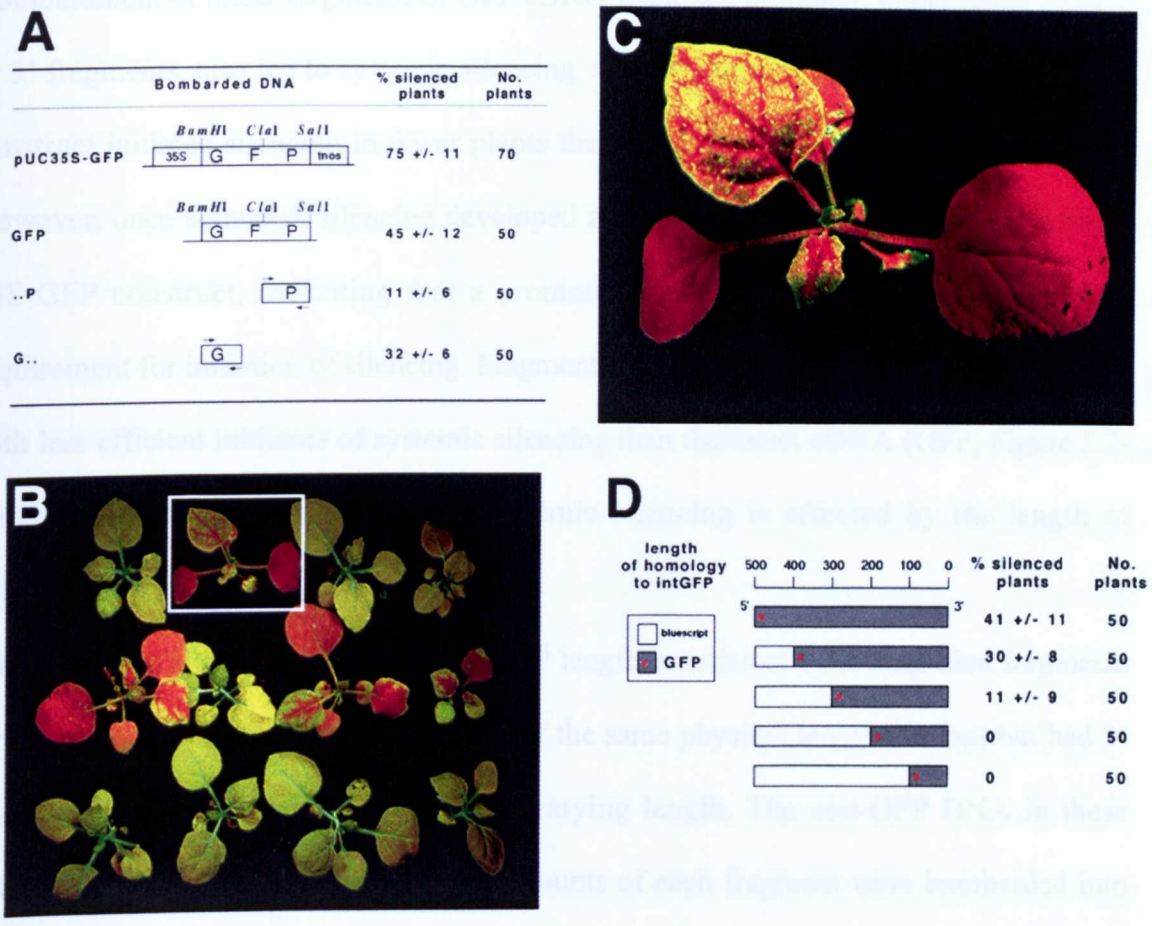

Figure 5.2. Biolistic activation of systemic silencing. (**A**) DNA constructs tested for biolistic activation of systemic silencing. The pUC35S-GFP plasmid contains the 35S-GFP expression cassette from pBin35S-GFP (Figure 5.1). The GFP plasmid contains only the full-length GFP open reading frame from pBin35S-GFP cloned as a *Bam*HI-*Sal*I restriction fragment in pUC19. The ..P and G.. DNA constructs are linear, PCR-amplified fragments of the GFP open reading frame and are respectively 348 and 453 bp long. Equal amounts of each construct were bombarded (see Experimental Procedures). (**B** and **C**) Silencing phenotype of intGFP bombarded plants. (B) A general view of a population of intGFP plants bombarded together 3 weeks previously with the promoterless GFP construct depicted in (A). (**C**) A close-up view of one of the silencing plants shows the characteristic vein-spreading of the systemic silencing signal, similar to that observed following *Agrobacterium* infiltration. (**D**) Effect of the length of homology between epiGFP and intGFP on biolistic activation of systemic silencing. The intGFP seedlings were bombarded with a series of PCR-amplified fragments sharing a similar physical length but harbouring 3' terminal fragments of GFP cDNA of varying length. These fragments were amplified from a pBluescript vector containing the full-length GFP open reading frame by using one vector-specific primer and one GFP-specific primer. The red dot on the diagram represents the 5' end of the GFP open reading frame. Equal amounts of each construct were bombarded (see experimental procedures).

Bombardment of linear fragments of GFP cDNA without a promoter, either intact or as 5' or 3' fragments, also led to systemic silencing. Overall, the full-length promoterless GFP construct initiated silencing in fewer plants than the 35S-GFP construct (Figure 5.2-A). However, once activated, silencing developed and spread as quickly as with the original 35S-GFP construct, indicating that a promoter upstream epiGFP is not an absolute requirement for initiation of silencing. Fragments of GFP (..P and G..; Figure 5.2-A) were both less efficient initiators of systemic silencing than the intact cDNA (GFP, Figure 5.2-A) thus indicating that initiation of systemic silencing is affected by the length of epiGFP.

To further investigate importance of epiGFP length, a series of PCR-amplified fragments were produced. These fragments were all of the same physical length (500bp) but had 3' co-terminal fragments of GFP cDNA of varying length. The non-GFP DNA in these fragments was from pBluescript. Equal amounts of each fragment were bombarded into 50 plants in 5 independent experiments. The results, summarised in Figure 5.2-D, clearly show that the efficiency of systemic silencing initiation is determined by the length of homology between the epiGFP and the intGFP sequences.

5.4. Propagation of the silencing signal

Symplastic movement of molecules in plants can occur from cell-to-cell through plasmodesmata and/or through the phloem (Lucas et al., 1993). To investigate which of these routes is used by the silencing signal, we monitored the spatial pattern of intGFP silencing after infiltration of plants with the epiGUS-epiGFP strain of *A. tumefaciens*. At

20 days post-infiltration of lower leaves, silencing was strongest in systemic, young developing leaves (Figure 5.3-A_4) and was very pronounced in the shoot tips (Figure 5.3-A_5), although the meristematic regions were still green fluorescent (Figure 5.3-A_5). There was also silencing in upper leaves that were already expanded at the time of infiltration but it was fainter and less extensive than in the young developing leaves (Figure 5.3-A_2 and A_3). In contrast, the leaves immediately above and below the infiltrated leaves remained fully green fluorescent (Figure 5.3-A_1). At 30 days post-infiltration the stem and roots below the infiltrated leaves also showed intGFP silencing, thus indicating that the movement of the silencing signal was bi-directional in the plant. In terms of speed and spatial distribution, this pattern of spread is similar to the movement of viruses in the phloem, from source to sink leaves (Leisner and Turgeon, 1993).

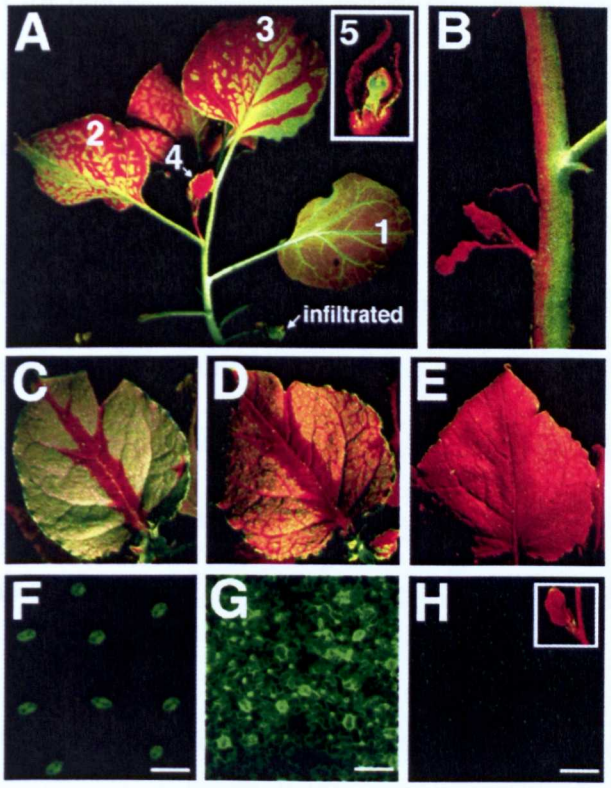

Figure 5.3. Phloem transport and cell-to cell movement of the systemic silencing signal. (**A**) Spatial pattern of systemic silencing at 20 days post-infiltration. The first fully expanded leaf above the infiltrated leaf is not affected by gene silencing (1), whereas upper source leaves that were expanding at the time of infiltration are partially affected (2, 3). Young developping sink leaves exhibit strong gene silencing (4). Panel 5 shows the apical bud of a GFP silenced plant. The young developing leaves are red fluorescent and fully silenced but the central growing point remains green fluorescent, indicating that GFP silencing is not active in this region. (**B**) Polar gene silencing following single leaf infiltration. One leaf of a intGFP plant was infiltrated with the epiGUS-epiGFP strain of *Agrobacterium*. After one month, distribution of intGFP gene silencing in the stem was monitored under UV illumination. In this example, the infiltrated leaf was connected to the left-hand side of the stem. (**C-E**) Development of silencing in leaves. intGFP silencing of a systemic leaf that had already expanded at the time of lower leaves infiltration was monitored under UV illumination. Each panel represents the same leaf at 3, 4 and 5 weeks post infiltration. (C-E, respectively). (**F-H**) Cell-to-cell movement of the systemic silencing signal. Confocal microscope observation of tissue samples taken from silenced leaves that had already expanded at the time of infiltration (such as the one depicted in D) shows that intGFP fluorescence is only evident in the symplastically isolated stomatal guard cells (E), whereas intGFP fluorescence is present in all leaf cells from non-silenced plant (F). In leaves that developed after the silencing had spread to the growing point (G, insert), intGFP fluorescence is absent even from guard cells (G). The bar represents 100 μm.

Additional support for phloem transport of the signal comes from experiments in which intGFP plants were infiltrated with the epiGUS-epiGFP strain of *A. tumefaciens* in just a single leaf. These experiments differ from those described previously in which the plants were infiltrated in two or three leaves on opposite sides of the plant. At one month post-infiltration, intGFP silencing in the stem was restricted to the side of the original infiltrated leaf (Figure 5.3-B). Shoots that had emerged from the silenced portion of the stem were silenced, while those emerging from the non-silenced half were not (Figure 5.3-B). This pattern of signal movement was strikingly similar to the spread of a phloem-translocated dye and of a systemic virus in *N. benthamiana* (Roberts et al., 1997).

The development of silencing in leaves was also similar to the translocation of a phloem-transported dye through class I, II and III veins of *N. benthamiana* leaves (Roberts et al., 1997). In systemic leaves that had already expanded at the time of infiltration, intGFP silencing was initially (20 days post infiltration) in regions surrounding the main veins (Figure 5.3-C) and later (27 days post-infiltration) in regions around the minor veins (Figure 5.3-D). At 34 days post-infiltration, intGFP silencing spread across the whole lamina of the leaf (Figure 5.3-E), indicating that there was cell-to-cell movement of the silencing signal as well as translocation through the phloem. This cell-to-cell movement is likely to occur through plasmodesmata because there was no intGFP silencing in the stomatal guard cells (Figure 5.3-F, G) which would have been symplastically isolated before the signal moved into the leaf. However, in leaves that developed after the signal had spread to the apical growing point, intGFP was uniformly silenced, even in the stomatal guard cells (Figure 5.3-H). From this observation, we conclude that guard cells

are competent for gene silencing provided that the signal invades leaves early in their

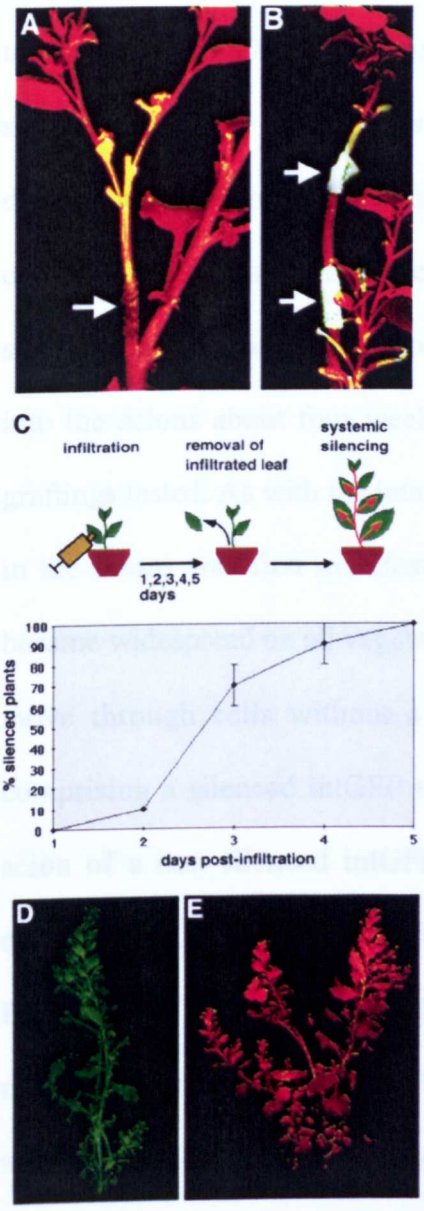

Figure 5.4 Graft transmission, kinetics of translocation, and persistence of the systemic silencing signal. All pictures were taken under UV light illumination. (**A-B**) Non-silenced intGFP scions were grafted onto silenced intGFP rootstocks, either directly (**A**) or with an nt stem section in between (**B**). In both instances, systemic silencing was transmitted to the intGFP scions, as shown by the loss of green fluorescence in upper parts of the graft. Residual intGFP in the scions appears yellow. Arrows indicate graft junctions. (**C**) Kinetics of translocation of the systemic silencing signal. The top diagram illustrates the order of events described below. One leaf of intGFP plant was infiltrated with the epiGUS-epiGFP strain of *A. tumefaciens* (previously induced with acetosyringone), and subsequently removed 1,2,3,4 or 5 days after infiltration. The percentage of plants undergoing systemic silencing after removal of the infiltrated leaf was then assessed under UV illumination. Each dot on the diagram represents the average percentage obtained from 30 individual plants infiltrated at the same time. (**D-E**) Persistence of systemic silencing. Silencing is efficiently maintained in intGFP plants that had been infiltrated more than 100 d previously (**E**). Water-infiltrated plants of the same age remained fully green fluorescent (**D**).

development, before symplastic isolation. To further investigate the movement of the silencing signal, we carried out grafting experiments that were specifically designed to determine whether the signal could move through cells in which there were no genes with sequence similarity to the target of silencing. Previous grafting experiments have reported

the transmission of a cosuppression state involving a nitrate reductase (NR) transgene and its corresponding endogenous homologue (Palauqui et al., 1997). It was shown that the silencing signal targetting the NR sequences in the transgenic rootstock could be transmitted through a non-transgenic section of plant. However, as this intermediate section carried the endogenous copy of the NR gene, it was still possible that long-distance transmission of the signal was dependent on homologous DNA or RNA. First, to confirm in our system that the signal is graft transmissible, we wedge-grafted non-silenced intGFP scions onto rootstocks exhibiting silencing of intGFP. Silencing spread into the scions about four weeks after the graft union (Figure 5.4-A) in 10 out of 16 graftings tested. As with the intact epiGUS-epiGFP infiltrated plants, intGFP suppression in the scions was first manifest around the veins of newly emerging leaves and later became widespread on all vegetative parts of the scions. To test the ability of the signal to move through cells without a GFP transgene, we then produced three-way grafts comprising a silenced intGFP rootstock, an intermediate section of nt stem and a top scion of a non silenced intGFP plant. Using this procedure, we observed silencing occurring in the intGFP top scions about six weeks after the graft junctions (Figure 5.4-B) in 5 out of 11 graftings tested. This result demonstrates that the silencing signal could move long distances and through cells in which there is no corresponding nuclear gene, as the intermediate section had no GFP sequence.

In a separate series of experiments, the speed of signal movement was assessed by removal of the infiltrated leaf 1, 2, 3, 4 or 5 days after infiltration with the epiGUS-epiGFP strain of *A. tumefaciens*. In these experiments, there was systemic loss of intGFP

fluorescence (i.e. silencing) in 10% of the plants if the infiltrated leaf was removed 2 days post-infiltration. A progressively higher proportion of plants exhibited systemic silencing when the infiltrated leaf was removed 3 days or later (Figure 5.4-C). From these data, we conclude that production and translocation of the signal occurs within 2 or 3 days post-infiltration.

In plants that exhibited silencing after removal of the infiltrated leaf, loss of intGFP developed as quickly and persisted for as long as in the intact plants. Furthermore, in all of the epiGUS-epiGFP-infiltrated plants, silencing of intGFP persisted for more than 100 days post infiltration. Even in these old plants (Figure 5.4-D, E), systemic silencing continued to be induced in the newly emerging leaves, despite the loss of the infiltrated leaf due to senescence. Considering these observations, we propose that propagation of the silencing signal occurs via a relay process. The cells receiving the signal from the infiltrated leaf would become a secondary source of the signal so that maintenance of PTGS in the plant would become independent of the infiltrated leaf.

5.5. Systemic silencing in meristems

Although there was extensive and persistent silencing of intGFP in the epiGUS-epiGFP-infiltrated *N. benthamiana* plants (as shown in Figure 5.4-E) the vegetative (Figure 5.3-A_5), floral and root apexes (data not shown) always remained non silenced (i.e. green fluorescent). Either the signal of gene silencing cannot enter dividing cells or dividing cells lack the potential to silence intGFP. To address these alternatives, we cultured leaf explants from plants exhibiting systemic silencing of intGFP. The explants were cultured

on media promoting shoot regeneration. It was expected that intGFP silencing would be lost if dividing cells lack the potential to silence intGFP.

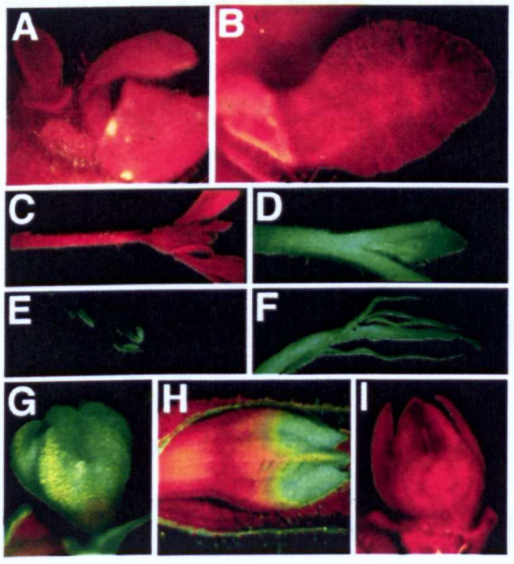

Figure 5.5. Systemic silencing is maintained through in vitro organogenesis but does not occur in the growing point. All pictures were taken under UV light illumination. The bar represents 1mm (A and B), 1cm (C and D), 0.7 cm (E and F), 0.7 mm (G-I). (**A-C**) Shoot regeneration from leaf disks excised from intGFP silenced plants. All vegetative parts of the shoots appear uniformly red, indicating that silencing is sustained through in vitro propagation. (**A**) shows leaves regenerated from intGFP silenced tissue. (**B**) is a close-up view of one regenerated leaf. (**C**) presents the stem and a flower bud of an explant regenerated from intGFP silenced tissue.

Figure 5.5-A-C shows shoots and leaves regenerating from these explants. Clearly, there was no intGFP fluorescence in most parts of these organs whereas shoots regenerated from non-silenced plants remained fully green fluorescent (Figure 5.5-D). From these observations we conclude that silencing was not induced by the culture procedures but that it could persist through in vitro organogenesis. However the extreme apical regions of the regenerated silenced shoots were green fluorescent, as in the progenitor plants. When the shoots developed into plants with roots, the root tips (Figure 5.5-E) and apical zones of vegetative (Figure 5.5-G) and floral (Figure 5.5-H) shoots were also green fluorescent. This apical fluorescence was not present in nontransformed plants (Figure 5.5-I and data not shown) and is therefore bona fide GFP rather than an artefact due to the presence of fluorescent compounds. These results indicate that silencing can be maintained in, or could pass through dividing cells during tissue regeneration but that the gene silencing mechanism is apparently not effective in meristematic tissues of the plant,

presumably because the silencing signal cannot reach those regions, or because these tissues lack a component that is necessary for perception of the signal. These findings reinforce the striking similarities between the movement of the signal and the movement of plant viruses (Figure 5.3), which are generally excluded from meristems (Matthews, 1991).

5.6. RNA turn-over mechanism activated upon reception of the systemic silencing signal

5.6.1 Systemic silencing is an example of sense suppression

The preliminary analysis described in chapter 4 showed that systemically silenced tissues exhibited sequence-specific resistance against challenged PVX-GFP. This indicated that a

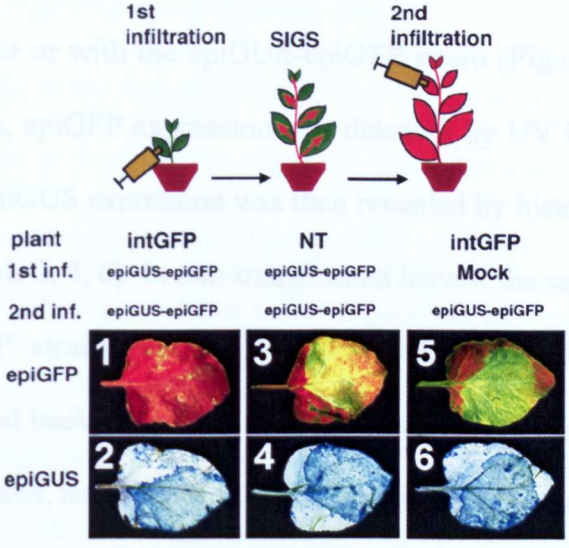

Figure 5.6. Secondary infiltrations of the epiGUS-epiGFP strain of *A. tumefaciens*. The top diagram illustrates the order of events described below. Lower leaves of intGFP plants (panels 1-2, 5-6) or nt plants (panel 3-4) were first infiltrated (1st inf.) with either water (panel 5-6) or the epiGUS-epiGFP strain of *A. tumefaciens* (panels 1-2, 3-4). After 20 days, an upper leaf was infiltrated with the epiGUS-epiGFP strain of *A. tumefaciens* (2nd inf.). Two days later, the leaf was monitored under U.V. illumination for transient epiGFP expression, and subsequently stained for epiGUS activity. The red fluorescence in panels 1 and 3 represents chlorophyll fluorescence. The green fluorescence which also appears yellow in some of these images represents expression of GFP. In panel 5, imaging of intGFP in the leaf lamina is partially masked by strong epiGFP expression, although intGFP is evident in the petiole of the leaf.

sequence-specific, antiviral mechanism had been activated in those tissues, which operates at the post-transcriptional level. These data also indicated that this mechanism is active in the cytoplasm, where PVX replicates and accumulates. This analysis, however, did not indicate whether the sequence-specific mechanism prevented replication of PVX-GFP or mediated an RNA degradation mechanism, as observed for PTGS of nuclear transgenes. Although the intGFP mRNA was reduced, it was thus still possible that the GFP transgene locus had been silenced at the transcriptional level in systemic tissues, with the signal merely acting as a trigger for this change as well as conferring protection against PVX-GFP.

To resolve these questions, a second agroinfiltration was carried out with the epiGUS-epiGFP strain in the upper leaves of intGFP plants that had been infiltrated 20 days previously with water or with the epiGUS-epiGFP strain (Figure 5.6, diagram). At two days post-infiltration, epiGFP expression was detected by UV illumination (Figure 5.6, panels 1, 3, 5) and epiGUS expression was then revealed by histochemical staining of the leaf (Figure 5.6, panels 2, 4, 6). In non-transformed leaves, the secondary infiltration with the epiGUS-epiGFP strain produced strong green fluorescence due to epiGFP superimposed on a red background due to chlorophyll fluorescence (Figure 5.6, panel 3) while in the transformed, non-silenced leaves there was strong green fluorescence due to the epiGFP superimposed over a green intGFP background (Figure 5.6, panel 5). These leaves also showed strong histochemical staining for GUS activity, indicating that the epiGUS gene was expressed (Figure 5.6, panels 4 and 6). However, on leaves exhibiting systemic silencing of intGFP, there was expression of the epiGUS reporter gene but not

of the epiGFP reporter (Figure 5.6, panels 1-2). This result shows that the expression of the introduced epiGFP but not the epiGUS transgene was affected in tissue exhibiting systemic silencing of intGFP and therefore confirms the sequence specificity of systemic silencing. In addition, as both epiGFP and epiGUS have a 35S promoter, this result confirms that systemic silencing is not targeted against the 35S promoter sequence. Furthermore, because a sense orientation intGFP transgene had been induced to target a sense orientation epiGFP, these data also show that systemic silencing is a sense suppression mechanism similar to the examples of PTGS in transgenic plants carrying sense transgenes.

5.6.2 The regions of intGFP RNA that are targeted in systemic tissues expand beyond the sequence of the triggering epiGFP construct.

We further characterised the targets of intGFP silencing in systemic tissues, following bombardments with 5' or 3' linear fragments of GFP cDNA (GF. and..P, Figure 5.7-A). If silencing was primed only against the epiGFP sequence, the target in the intGFP mRNA would be confined to the region corresponding to the bombarded DNA. Alternatively, targeting could extend beyond the regions of the bombarded DNA. The assay for silencing target sites involved inoculation of PVX-GF and PVX-P (Figure 5.7-A) to intGFP plants that had been bombarded 21d previously with GFP, ..P or GF. (Figure 5.7-B). Virus inoculations were made to leaves exhibiting systemic silencing of intGFP and accumulation of the viral RNA was assessed by northern analysis of RNA samples taken from the inoculated leaves at 8d post inoculation (Figure 5.7-B).

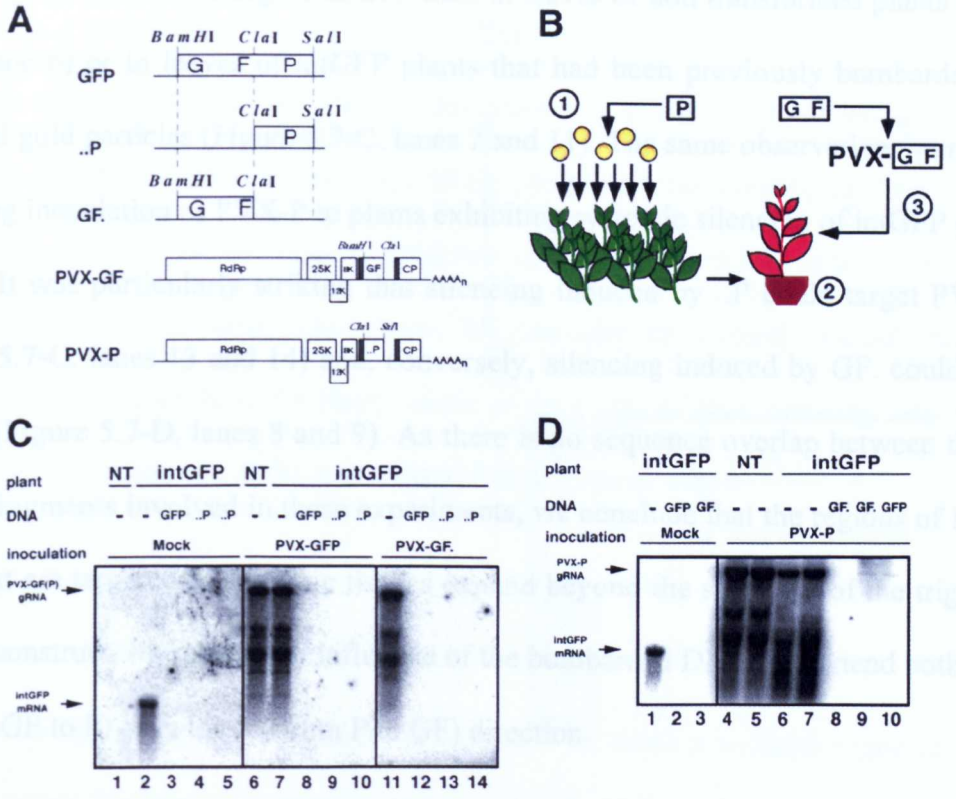

Figure 5.7. The regions of intGFP RNA that are targeted in systemic tissues expand beyond the sequence of the triggering epiGFP construct. (**A**) Bombarded epiGFP and inoculated viral constructs. The ..P and GF. DNA constructs are derivatives of the GFP construct described in Figure 4A. PVX-GF and PVX-P are PVX vectors carrying the GF. and ..P restriction fragments of the GFP open reading frame, respectively. Expression of the corresponding insert is controlled by a duplicated coat protein (CP) promoter indicated by shaded boxes (RdRp: RNA dependent RNA polymerase, 25K, 12K, 8K: cell-to-cell movement proteins, CP:coat protein). (**B**). Diagram illustrating the order of events described below. (**C**) Northern analysis of intGFP and PVX-GF/GFP RNAs. First, intGFP seedlings of nt plants were bombarded with either uncoated gold particles (-) or gold particles coated with either the GFP or the ..P construct (see panel B①). After 21 days, when intGFP was systemically silenced (see panel B②), two upper leaves were inoculated with either water (Mock), PVX-GFP or PVX-GF (see panel B③). Five days after virus inoculation, total RNA was extracted from one of the two inoculated upper leaves and Northern analysis of 10 μg of RNA was carried out to detect accumulation of the intGFP and PVX-GF/GFP RNA (indicated on the left side of the upper panel). (**D**) Northern analysis of intGFP and PVX-P RNAs. The analysis was performed as described in (C), following inoculation of PVX-P to GF-bombarded intGFP plants.

Northern analyses of inoculated leaves showed that accumulation of PVX-GFP and PVX-GF (Figure 5.7-C, lanes 8-10 and 12-14) was lower (by at least ten fold) in leaves exhibiting systemic silencing of intGFP than in leaves of non transformed plants (Figure 5.7-C lane 6) or in leaves of intGFP plants that had been previously bombarded with uncoated gold particles (Figure 5.7-C, lanes 7 and 11). The same observations were made following inoculation of PVX-P to plants exhibiting systemic silencing of intGFP (Figure 5.7-D). It was particularly striking that silencing induced by ..P could target PVX-GF (Figure 5.7-C, lanes 13 and 14) and, conversely, silencing induced by GF. could target PVX-P (Figure 5.7-D, lanes 8 and 9). As there is no sequence overlap between the GF. and ..P fragments involved in these experiments, we conclude that the regions of intGFP RNA that are targeted in systemic tissues expand beyond the sequence of the triggering epiGFP construct. Moreover, the influence of the bombarded DNA can extend both in the 3' (from GF to P) or in the 5' (from P to GF) direction.

5.7 Discussion

As done in a previous analysis of VIGS (Chapter 3), we describe in this chapter the dissection of systemic silencing into separate initiation, systemic spread and maintenance stages. In this discussion we assess the likely molecular mechanisms of these different stages, the possible natural role of systemic silencing as well as related phenomena observed in organisms other than plants. We consider the systemic spread stage first, because the inferences about the likely nature of the signal of gene silencing influence the subsequent discussion about the initiation and maintenance stages.

5.7.1 Systemic spread of PTGS

Systemic spread of PTGS is remarkable in that it involves a sequence-specific signal: systemic silencing initiated against GFP was specific for intGFP or viral GFP RNAs, whereas it was shown in other work that systemic silencing against GUS or the nitrate reductase was specific for GUS or nitrate reductase (NR) RNAs. This pattern of sequence specificity rules out the possibility that this systemic silencing is a non-specific wounding signal or that the specificity is related to the 35S promoter. Therefore it is likely that the signal of silencing is specific for the transcribed regions of the target gene and that the specificity determinant includes a nucleic acid component. Thus, the signal for silencing of GFP is likely to contain GFP RNA or DNA, whereas the signal for silencing of GUS or other genes would contain the corresponding alternative nucleic acid species. From its pattern and speed of systemic spread, we confirm that this putative nucleic acid is able to

move not only from cell to cell through plasmodesmata but also systemically through the phloem, as speculated by others (Jorgensen et al., 1998).

There are precedents in plants for endogenous nucleic acids that move between cells. For example, there are mobile nucleic acids encoded by nuclear genes including the mRNA for a transcription factor (Lucas et al., 1995) and a sucrose transporter mRNA (Kuhn et al., 1997). However, in both of these examples the movement is only between cells: there is no evidence for long distance movement, as with the silencing signal. The mobile nucleic acids that are most obviously comparable to this putative signal are viroids. Like the signal of silencing, these small non-coding RNA species move systemically within a period of a few days after inoculation (Palukaitis, 1987). For both viroids and the silencing signal, the route of movement involves cell-to-cell through plasmodesmata and long distance spread through the phloem (Ding et al., 1997; Palukaitis, 1987). As for movement of viroids, trafficking of the silencing signal may be dependent on host-encoded proteins. One possible candidate for a silencing signal trafficking factor is CmPP16 from *Cucurbita maxima* (XoconostleCazares et al., 1999). This protein has limited sequence homology with the movement protein of red clover necrotic mosaic virus (RCNMV) and possesses the capacity to move from cell-to-cell and to mediate transport of sense and antisense RNA. Moreover, in grafting experiments, CmPP16 moves together with its mRNA into the sieve elements of scion tissue (XoconostleCazares et al., 1999).

From the leaf detachment experiment we infer that movement of the signal involves a relay. Some cells receiving the epiGFP were the primary source of initial signal production. However, once the signal moved out of the bombarded or infiltrated area this primary source was no longer required and there must have been cells elsewhere in the plant providing a secondary source of the signal molecule. We do not know the maximum distance between primary and secondary relay points in signal production but from the three-way grafting experiments we can infer that distances of several centimetres or more could be involved.

5.7.2. Initiation and maintenance of signal production

Systemic silencing was initiated in the bombarded or infiltrated cells that received epiGFP. It is unlikely that initiation of systemic silencing required high levels of transcription of the introduced DNA because the presence of a promoter upstream of the GFP sequences had little effect in the bombardment experiments. Moreover, a similar conclusion was drawn from experiments in which *Agrobacterium*-mediated systemic silencing was activated with promoterless GFP constructs engineered in the pBin19 T-DNA (data not shown). Consistent activation of systemic silencing of a NR transgene by bombardment of promoterless, homologous DNA has also been reported elsewhere (Palauqui and Balzergue, 1999). The involvement of transcription cannot be excluded, however. Unintended transcription could result from integration of some of the transferred T-DNA or bombarded DNA molecules downstream of endogenous promoters. Even if these events are rare, the probable high amount of individual DNA

molecules transferred into the nucleus make it possible that epiGFP RNA species are produced from promoterless constructs.

We do not favour the idea that the signal was derived directly from the introduced epiGFP DNA or putative RNA species because systemic silencing induced by ..P resulted in targeting of the GF. component of intGFP RNA. Similarly, bombardment of GF. produced silencing targeted against ..P. Our interpretation of these data is that silencing was initiated by an interaction between intGFP and epiGFP and that the target of systemic silencing was determined by intGFP. The influence of epiGFP length on silencing is also consistent with a homology-dependent interaction between epiGFP and intGFP. This effect of length has also been reported for activation of systemic silencing of NR in transgenic tobacco (Palauqui and Balzergue, 1999).

It is likely that this proposed interaction induces an epigenetic modification of transgene DNA and that this change is necessary for systemic signalling to occur. For instance, in the NR transgenic system, it is clear that not all NR transgene loci are equally responsive to the same bombarded DNA trigger of systemic silencing, despite the fact that these transgene loci produce similar, high levels of NR RNA (Palauqui and Vaucheret, 1998). The difference suggests that a DNA feature carried by some transgene loci allows them to respond to the bombarded molecules and acquire competence to initiate systemic silencing. Thus, a modification at the DNA level, induced by the ectopic DNA or RNA, must be invoked in this case. An analysis revealed that *de novo* methylation of the intGFP trangene coding region occurs in epiGFP-infiltrated tissues (Jones et al., 1999). This

observation is therefore consistent with the proposal that initiation of systemic silencing requires epigenetic modification of transgene DNA. It would be of great interest to compare the methylation status of the NR transgenic loci that prevent or allow initiation of systemic silencing in tobacco.

How could a modification occurring at the DNA level result in systemic silencing? We propose here a mechanism similar to an earlier ectopic pairing model of PTGS in transgenic plants (Baulcombe and English, 1996). However, we currently consider that the DNA level interaction could involve DNA:DNA pairing or RNA:DNA pairing. According to this proposed mechanism, the ectopic interactions of epiGFP DNA/RNA and intGFP DNA would perturb transcription of the intGFP and lead ultimately to formation of an aberrant RNA. It is possible that this molecule is similar to the maintenance RNA-containing factor that was evoked in Chapter 3 to explain the persistence of GFP VIGS in tissues where PVX-GFP had been eliminated. The proposal that there could be ectopic interactions of homologous DNA leading to aberrant transcription is based on precedents from plants, animals and fungi. In one example, with ß-globin genes in mammalian cells, an ectopic DNA interaction was demonstrated directly by the co-localisation of a transfected plasmid with the homologous sequence in the genome (Ashe et al., 1997). In plant and fungal cells, the ectopic interaction could only be inferred indirectly from the modified methylation pattern of the homologous DNAs (Barry et al., 1993; Hobbs et al., 1990). We envisage that these ectopic interactions may lead to aberrant RNA either by arrest of transcription leading to prematurely truncated RNA species, as shown in *Ascobolus immersus* (Barry et al., 1993).

Alternatively the ectopic interactions could cause aberrant extension of transcription, as in the example with ß-globin genes (Ashe et al., 1997). These two possibilities are not mutually exclusive.

If aberrant RNA includes negative strand sequence, it could target the sense intGFP RNA for degradation. Alternatively, the aberrant RNA could be recognised and processed by a surveillance system that would activate sequence-specific intGFP RNA degradation. This surveillance/processing system could involve a host-encoded RNA-dependent RNA polymerase (RdRp), as suggested originally to explain sense transgene-mediated PTGS (Lindbo et al., 1993). In this scenario, the RdRp could produce a dsRNA using the aberrant sense RNA as template. The resulting molecule or RNA species derived from it could then be part of the systemic signal. As discussed in Chapter 3, dsRNA appears to be a potent initiator of PTGS in plants and other organisms. Thus, production of such molecule could also explain the intracellular silencing activated by epiGFP (Chapter 4, Figure 4.2).

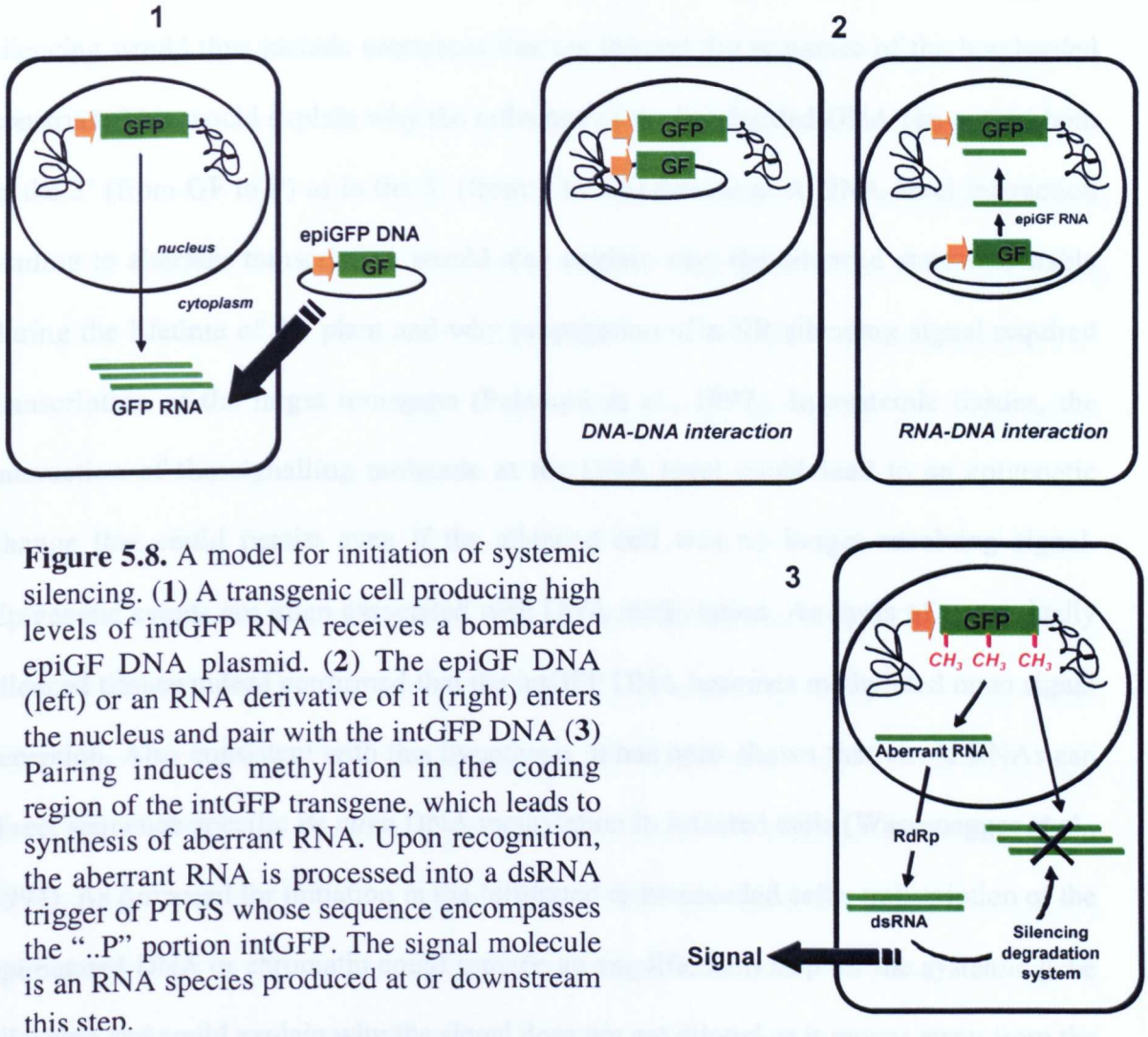

Figure 5.8. A model for initiation of systemic silencing. (**1**) A transgenic cell producing high levels of intGFP RNA receives a bombarded epiGF DNA plasmid. (**2**) The epiGF DNA (left) or an RNA derivative of it (right) enters the nucleus and pair with the intGFP DNA (**3**) Pairing induces methylation in the coding region of the intGFP transgene, which leads to synthesis of aberrant RNA. Upon recognition, the aberrant RNA is processed into a dsRNA trigger of PTGS whose sequence encompasses the "..P" portion intGFP. The signal molecule is an RNA species produced at or downstream this step.

This proposal is attractive because the aberrant RNA produced from ectopic interaction between epiGFP RNA/DNA and intGFP DNA could extend beyond the sequences of GF. or ..P. If the aberrant RNA is a template for an RdRp, the resulting dsRNA trigger of silencing would thus include sequences that are beyond the sequence of the bombarded construct. This would explain why the influence of the bombarded DNA can extend both in the 3' (from GF to P) or in the 5' (from P to GF) direction. A DNA-level interaction leading to aberrant transcription would also explain why the silenced state was stable during the lifetime of the plant and why propagation of a NR silencing signal required transcription of the target transgene (Palauqui et al., 1997). In systemic tissues, the interaction of the signalling molecule at the DNA level could lead to an epigenetic change that could persist even if the silenced cell was no longer receiving signal. Epigenetic events are often associated with DNA methylation. Analysis of systemically silenced tissues indeed confirmed that the intGFP DNA becomes methylated upon signal reception. Also consistent with this hypothesis, it has been shown that viroid RNAs can direct sequence-specific *de novo* DNA methylation in infected cells (Wassenegger et al., 1994). As proposed for initiation in the infiltrated or bombarded cells, transcription of the epimutated DNA or chromatin could provide an amplification step for the systemic gene silencing and could explain why the signal does not get diluted as it moves away from the sites of initiation.

5.7.3 Systemic silencing compared to other examples of gene silencing in plants and animals

Many examples of gene silencing in plants may be similar to the phenomenon described here. For example, in transgenic plants exhibiting transgene-induced PTGS, it is clear from grafting experiments (Palauqui et al., 1997) and from the spatial patterns of silencing (Kunz et al., 1996) that PTGS is a non cell-autonomous event. In addition we consider it likely that gene silencing with a delayed onset, for example with GUS transgenes, may also involve systemic spread of a signal (Elmayan and Vaucheret, 1996). In these instances, we envisage that the process may be initiated in just one or a few cells in the plant, as shown here in systemic silencing, and that the spread of the signal accounts for the gene silencing throughout the plant.

The involvement of a signal molecule means that genetic or epigenetic variations in single cells could influence the level of gene silencing throughout the plant. Consequently, the analysis of transgenes in whole plant DNA may not be an accurate indicator of factors that influence PTGS. For example, in a previous study based on analysis of whole plant DNA, it was concluded that single copy, hemizygous transgenes can activate PTGS (Elmayan and Vaucheret, 1996). This conclusion was difficult to reconcile with the suggestion that ectopic DNA interactions initiate PTGS (Baulcombe and English, 1996). However, the results presented here show that the PTGS in the whole plant could have been initiated in individual cells carrying multiple copies of the transgene due to DNA endoreduplication or chromosomal rearrangements. Therefore,

even in plants having only one copy of a silencer transgene in the genome, it cannot be ruled out that PTGS was initiated by ectopic interactions of homologous DNA.

There are now reports of gene silencing phenomena in animals that appear similar, at least superficially, to systemic silencing in plants. The RNA interference described initially in *C. elegans* (Fire et al., 1998) and subsequently in many other organisms (reviewed in (Hammond et al., 2001)) is initiated by dsRNA rather than DNA, as described here, but otherwise shares many common features with the systemic silencing, including the ability to spread through the affected animal. Minute amount of dsRNA are required to trigger RNAi in a finite number of cells. However, the entire organism is eventually affected (Fire et al., 1998). Thus, it is likely that spread of RNAi involves a form of relay process which may be similar to that described in the GFP plants. In *Paramecium*, microinjection of plasmids containing sequences of a gene leads to homology-dependent silencing of the corresponding gene in the somatic macronucleus (Ruiz et al., 1998). As described here, the silencing effect could be initiated with plasmids containing only the coding region of the gene and was stably maintained throughout vegetative growth of the organism. Perhaps the similarity between systemic silencing in plants, the induced silencing in *Paramecium* and the effect of double stranded RNA in *C. elegans* reflects the existence of a ubiquitous mechanism in plants and animals that is able to specifically target aberrant RNA. This possibility fits well with the suggestion that RNA double-strandedness is a possible aberration required for initiation of PTGS in transgenic plants.

5.7.4. A natural role for systemic silencing in plants?

We have discussed in Chapter 3 the various lines of evidence that PTGS is part of a defence mechanism against virus infection. Our findings that the silencing signal (i) can be initiated at the single cell level, (ii) is rapidly translocated to remote parts of the plant and (iii) recruits the same channels as those used by most plant viruses, suggest that the silencing signal could represent the systemic arm of the PTGS defence. We consider it possible that an antiviral signal could be initiated in primary infection sites and could move ahead of the inducing virus. Consequently, before the virus would have moved systemically, a wave of antiviral gene silencing would already travel through the plant and would later delay the spread of the infection front. Because in natural infections this signalling would occur in the absence of virus-homologous DNA, we anticipate that some features of the putative virus-induced systemic signalling may differ from those associated with transgene-induced systemic silencing. For instance, the maintenance and relay properties that result from epigenetic modifications of DNA may not be observed.

It is also possible that systemic silencing represents a natural signalling mechanism invoved in plant development and physiology. For example, it is known from classical experiments that there is a graft transmissible signal of flowering (florigen) which has many of the predicted attributes of a natural manifestation of systemic silencing (Poethig, 1990). Like the silencing signal, florigen does not correspond to any of the conventionally characterised hormones or other signalling molecules in plants but it does move systemically to produce an epigenetic change (Bernier, 1988). With florigen, the epigenetic change is associated with the transition from the vegetative to the flowering

state of the plants while in systemic silencing it is associated with suppression of gene expression.

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CHAPTER 6

Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*

6.1 Abstract

Post-transcriptional gene silencing (PTGS) of a green fluorescent protein (GFP) transgene is suppressed in *Nicotiana benthamiana* plants infected with potato virus Y (PVY) or with cucumber mosaic virus (CMV) but not in plants infected with potato virus X (PVX). By expressing PVY and CMV-encoded proteins in a PVX vector it is shown that the viral proteins responsible for silencing suppression are the HCPro of PVY and the 2b protein of CMV. The HCPro acts by blocking the maintenance of PTGS in tissues where silencing had already been set, whereas the 2b protein prevents initiation of gene silencing at the growing points of the plants. Combined with the previous findings that viruses are both activators and targets of PTGS, these data strongly support the idea that PTGS represents a natural mechanism for plant protection against viruses. Accordingly, plant viruses have evolved strategies to overcome this defence.

6.2 Introduction

As shown in the previous chapters, PTGS of transgenes can be targeted against viral RNA and, extrapolating from this finding, it has been proposed that PTGS is a manifestation of a natural virus resistance mechanism in plants (Baulcombe, 1996; Pruss et al., 1997). According to this idea, PTGS is activated in transgenic plants when a transgene, or its RNA, is perceived as a virus. In support of the proposed relationship between PTGS and natural virus resistance, I have shown that PVX is an activator as well as a target of silencing, provided that there is sequence-homology with a nuclear (trans)gene (Chapter 3). Moreover, caulimo- and nepoviruses induce a PTGS-like resistance mechanism even if there is no sequence similarity between the virus and nuclear genes (Covey et al., 1997; Ratcliff et al., 1997). This mechanism causes the systemically-infected leaves to be symptom-free, to have only low levels of the virus and to have RNA sequence-specific resistance against challenge virus infection. Finally, the work of Ratcliff and colleagues provided direct evidence that, in wild-type *N.benthamiana,* PTGS is a defence response against PVX and TRV, two unrelated plant viruses (Ratcliff et al., 1999).

If there is a natural PTGS-like virus resistance in plants, it is likely that viruses would evolve strategies to avoid or suppress this mechanism. This idea was first developed based on analysis of plants infected with two viruses in which the disease symptoms were more severe than in plants infected with either of the two viruses alone (Pruss et al., 1997). In plants infected with a potyvirus this synergism was due to suppression of a host defence mechanism by the P1-HC-protease (Pruss et al., 1997). Following from this discovery, it was suggested that P1-HCPro is targeted against a PTGS-like resistance mechanism.

A second candidate suppressor of a PTGS-like resistance mechanism is the 2b protein encoded in cucumber mosaic virus (CMV) (Ding et al., 1995). This protein is required for long distance transport of CMV (Ding et al., 1995) and is now thought to act by suppressing a host resistance. In the absence of a suppressor, the resistance mechanism would prevent entry, translocation or exit of CMV from the phloem of infected plants. Conceivably, this resistance mechanism could also rely on a PTGS-like mechanism.

Here, the hypothesis that the P1-HCPro and 2b proteins are suppressors of PTGS is tested through the use of the GFP silencing system described in chapters 4 and 5. Transgenic GFP-silenced plants were infected with a potyvirus (PVY) and with CMV. Silenced plants were also infected with PVX and with chimaeric constructs carrying coding sequences from PVY and CMV in a PVX vector. If PVY or CMV produce suppressors of a PTGS-like resistance mechanism we predicted that infection by PVY, CMV or the PVX vectors would interfere with PTGS. The outcome of these experiments was consistent with this prediction and reveals that the HC-protease (HCPro) and the 2b protein suppress different stages of the systemic silencing mechanism, as characterised in chapter 5. These results implicate a PTGS-like mechanism as a limiting factor in the accumulation and spread of PVY, PVX and CMV. Moreover, as these are unrelated viruses, these findings support the suggestion that PTGS is a generalised anti-viral defence in plants.

6.3. Reversion of GFP silencing by wild-type PVY

From the analysis of plants infected with a potyvirus and a second virus, it had been shown that potyviruses encode a suppressor of a host plant defence against virus infection. To investigate the relationship of this defence mechanism to PTGS we inoculated PVY, the type-member of the potyviridae, to transgenic *N. benthamiana* exhibiting PTGS of a GFP

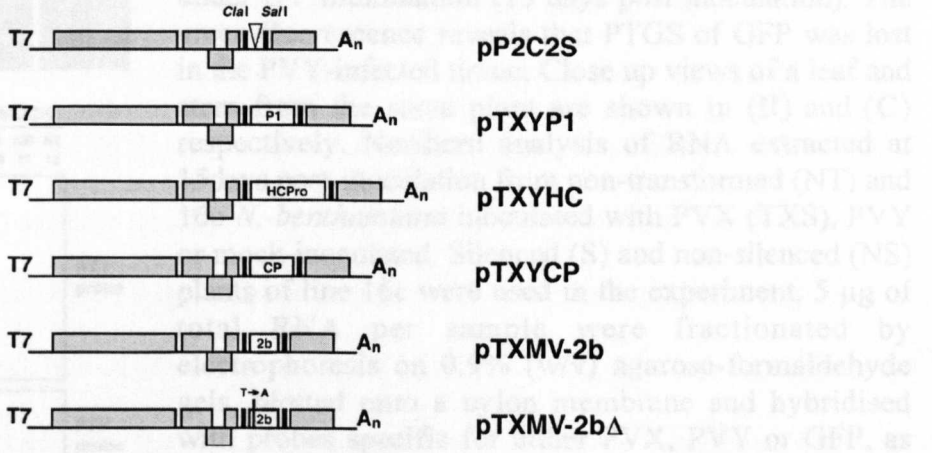

Figure 6.1 Schematic representation of the PVX vector and chimaeric constructs incorporating PVY and CMV coding sequences. Individual sequences were inserted into the pP2C2S PVX vector using the *Cla*I and *Sal*I cloning sites. The inserted PVY sequences are labelled: P1, 31 kD amino-terminal protein; HCPro, helper component protease; NIb, nuclear inclusion b; CP, coat protein. 2b refers to the CMV RNA 2-encoded protein Mutant versions of the HCPro and the 2b open reading frames were also introduced into the PVX vector. In the pTXYHCΔ construct there was -1 frame shift in the first codon of the HCPro open reading frame (Δ1). The pTXMV-2bΔ construct had a single nucleotide substitution (T→A) that converted the fourth codon (T<u>T</u>G) of the 2b open reading frame to a stop codon (T<u>A</u>G).

transgene. We predicted that there would be reversion of GFP silencing in the PVY-infected tissues if the suppressed defence mechanism is related to PTGS.

PTGS of the GFP transgene was induced by infiltration of lower leaves of 3 weeks-old seedlings with the epiGFP strain of *Agrobacterium tumefaciens*, as described previously (Chapter 5, section 5.3.1). This strain carries a binary-Ti plasmid containing the same GFP expression cassette that was used for plant transformation (epiGFP, chapter 5). We showed previously that silencing of GFP is initiated in the infiltrated zone and that a systemic GFP silencing signal spreads through the plant. Eventually the plant appears completely red under UV light due to sequence-specific, post-transcriptional gene silencing of the GFP transgene.

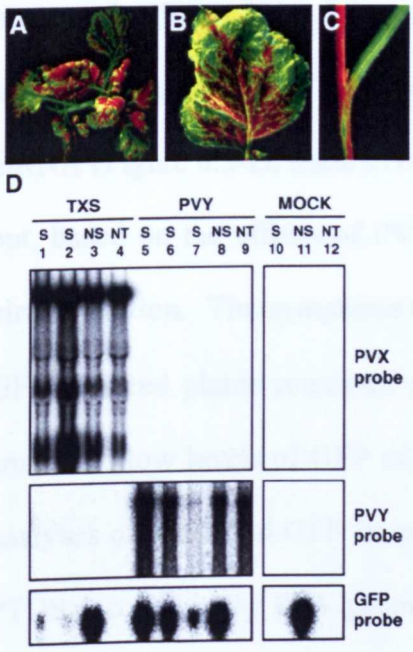

Figure 6.2. Suppression of PTGS by PVY. (**A**) GFP-silenced *N. benthamiana* line 16c infected with PVY under UV illumination (15 days post inoculation). The green fluorescence reveals that PTGS of GFP was lost in the PVY-infected tissue. Close up views of a leaf and stem from the same plant are shown in (**B**) and (**C**) respectively. Northern analysis of RNA extracted at 15days post-inoculation from non-transformed (NT) and 16c *N. benthamiana* inoculated with PVX (TXS), PVY or mock-inoculated. Silenced (S) and non-silenced (NS) plants of line 16c were used in the experiment. 5 μg of total RNA per sample were fractionated by electrophoresis on 0.9% (w/v) agarose-formaldehyde gels, blotted onto a nylon membrane and hybridised with probes specific for either PVX, PVY or GFP, as indicated.

To test for a suppressor of gene silencing, PVY was inoculated two weeks after infiltration when systemic gene silencing was complete in all tissues of the plants, except in the extreme meristematic zones that always remains non-silenced (Chapter 5, section 5.5). By two weeks post-inoculation, the GFP-silenced plants showed the systemic mild mottle and leaf curling symptoms of PVY, indicating that the virus had spread from the inoculated leaf. Under UV light, there were large regions of GFP fluorescence coinciding with the viral symptoms (Figure 6.2-A-C). Northern analysis of RNA extracted from these plants showed that the

effects on GFP fluorescence were parallel to the levels of GFP mRNA. Thus, in mock-inoculated GFP-silenced plants (Figure 6.1-D, track 10) the GFP mRNA levels were below the limit of northern blot detection, whereas in plants infected with PVY, the levels were similar to those in non-silenced plants (Figure 6.2-D, tracks 5, 6 and 11). One of the samples from a PVY-infected plant had only low level of GFP mRNA (Figure 6.2-D, track 7). However in this sample there was also only a low level of PVY RNA indicating a relationship between the levels of GFP mRNA and of PVY. We could rule out that these increased GFP mRNA levels were due to a non-specific enhancement of transgene expression because PVY infection in non silenced plants had no effect on the level of GFP mRNA (Figure 6.2-D, track 8) or on GFP fluorescence (data not shown). We could also rule out, based on the effects of PVX, that reversion of silencing was a non-specific effect of virus infection. The symptoms of PVX are a mild mosaic like those of PVY. However the GFP-silenced plants remained red-fluorescent after PVX infection (data not shown) and contained low levels of GFP mRNA (Figure 6.2-D, tracks 1, 2 and 3). Therefore, from the analyses of RNA and GFP fluorescence, these results were consistent with a suppressor of PTGS encoded in the PVY genome.

6.4. Reversion of GFP silencing by PVX-PVY recombinant viruses

The amino-terminal P1 and HCPro potyviral proteins have been implicated in suppression of host defence. In order to test the role of these proteins in the suppression of gene silencing, a series of PVX vectors carrying PVY gene sequences were inoculated to *N. benthamiana* plants exhibiting systemic PTGS of GFP. All these PVX vectors were constructed and described previously (Gianinna Brigneti's thesis). Here, they are named according to the

PVY-encoded protein produced in the infected plants. Thus, the pTXYHC vector produces HCPro: pTX refers to the backbone of these constructs which is a full length cDNA of PVX and Y refers to PVY. The plasmids carrying these constructs are pTXY**, where ** identifies the PVY protein. The viruses produced when the transcripts of these plasmids were inoculated are simply TXY**.

Most of these TXY** viruses induced mosaic symptoms, like the wild type PVX (data not shown). However, TXYHC produced symptoms that were much more severe than those of wild type PVX, inducing necrosis in stems and leaves, in addition to stunting of the infected plants (Figure 6.3-A and C), as described previously for a PVX construct expressing the HCPro of tobacco etch virus (TEV).

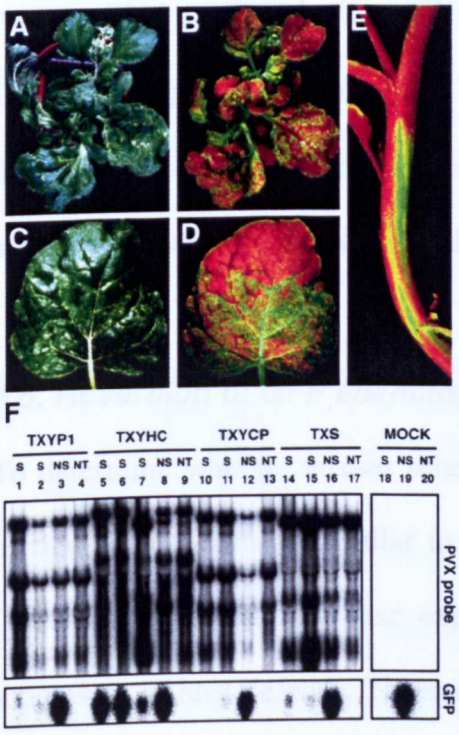

Figure 6.3. Suppression of PTGS by TXYHC. (**A**) GFP-silenced *N. benthamiana* (line 16c) illuminated under white light showing symptoms of TXYHC infection (15d post inoculation) The same plant, shown under UV illumination in (**B**), revealing that PTGS of GFP was suppressed in all symptomatic tissues infected with the chimaeric virus. (**C**) Close up of a leaf from the same plant under white light. (**D**) Close up of the same leaf under UV illumination showing the co-localization of GFP expression with viral symptoms. (**E**) Close up of the stem. (**F**, **G**) Northern analyses of RNA extracted at 15d post inoculation from non-transformed (NT) and *N. benthamiana* (line 16c) inoculated with PVX (TXS), TXY** recombinant viruses or mock-inoculated. Silenced (S) and non-silenced (NS) plants of line 16c were used in the experiments. 5 μg (F) or 1 μg (**G**) of total RNA per sample were fractionated by electrophoresis on 0.9% (w/v) agarose-formaldehyde gels, blotted onto nylon membranes and hybridised with probes specific for PVX or GFP RNAs, as indicated.

The TXYP1 and TXYCP viruses were similar to PVX (TXS) in that they had no effect on GFP silencing. Under UV illumination, at two weeks post inoculation, GFP-silenced plants infected with these constructs remained red, indicating that there had been no suppression of GFP gene silencing. Correspondingly, the GFP mRNA levels remained low in these plants (Figure 6.3-F, tracks 1-4 and 10-13). However, in TXYHC-infected plants, there were large green fluorescent regions coinciding with the viral symptoms (Figure 6.3-E). This effect was evident in all infected tissues, including stems (Figure 6.3-E) and leaves (Figure 6.3-C and D) that were already developed at the time of virus inoculation. Northern blot analysis showed that GFP mRNA levels had increased in these green fluorescent tissues and that the amount of GFP mRNA correlated with the relative amounts of TXYHC present in the sample (Figure 6.3-F, tracks 5-9). To rule out that the effect of TXYHC on gene silencing was an RNA-mediated effect TXYHCΔ was constructed (this clone was produced by Giannina Brigneti, Sainsbury Lab). TXYHCΔ carries a frame-shift mutation at the 5' end of the HCPro insert and did not suppress GFP gene silencing. The symptoms of TXYHCΔ were mild, like those of wild type PVX (data not shown).

6.5. Reversion of GFP silencing by wild type CMV

To determine whether viruses other than potyviruses can suppress gene silencing, we carried out experiments similar to those described above with CMV instead of PVY. CMV was selected for these experiments because, like potyviruses, it encodes a suppressor of host defence. Three weeks post-inoculation there were mosaic symptoms on the upper leaves of the plants. Under UV light, the symptomatic leaves of the GFP-silenced plants remained red-fluorescent if they had emerged from the growing points

before virus infection. However, the leaves emerging from the growing points after systemic spread of the virus became bright green fluorescent (Figure 6.4-A). There was no effect of CMV on the GFP fluorescence of non-silenced plants (data not shown). Northern analysis revealed that the GFP fluorescence in the CMV-infected plants was correlated with the levels of GFP RNA: the red fluorescent leaves of the silenced plants had GFP RNA levels that were below the limit of detection (Figure 6.4-B, track 4) whereas in the newly emerging tissues that were green fluorescent, GFP RNA levels were similar to those in non-silenced plants (Figure 6.4-B, tracks 5 and 6). The levels of GFP RNA on non-silenced plants were unaffected by CMV infection (Figure 6.4-B, tracks 3 and 6). From these results, we conclude that CMV encodes a suppressor of PTGS. However, because CMV and PVY do not suppress silencing in the same parts of the plant, these viruses must block different stages in the gene silencing mechanism.

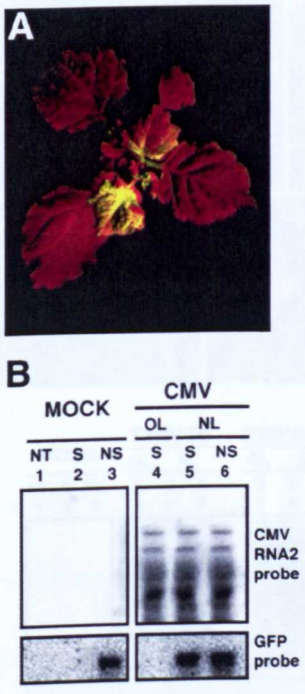

Figure 6.4. Suppression of PTGS by CMV. (**A**) GFP-silenced *N. benthamiana* (line 16c) infected with CMV (21d post inoculation). GFP expression was restored in the newly emerging tissue after systemic CMV infection had been established. (**B**) Northern analysis of RNA extracted at 15d post CMV inoculation from silenced (S) and non-silenced (NS) *N. benthamiana* plants of line 16c and from non-transformed plants. These plants were either infected with CMV or were mock inoculated. The RNA samples were taken either from old leaves (OL) that had emerged before systemic CMV infection or from new leaves (NL) emerging after CMV had spread systemically. 5 μg of total RNA per sample were fractionated by electrophoresis on a 0.9% (w/v) agarose-formaldehyde gel, blotted onto a nylon membrane and hybridised with probes specific for RNA2 of CMV or for GFP RNA, as indicated. The multiple RNA species hybridising to the CMV probe may represent degraded or subgenomic RNAs and have been described previously.

6.6. Reversion of GFP silencing by PVX-CMV recombinant viruses

The putative CMV-encoded suppressor of host defence is the 2b protein. By analogy with the potyviral HCPro, we considered that the ability of the 2b protein to suppress a plant defence mechanism could be due to its ability to suppress PTGS. To test this hypothesis, I

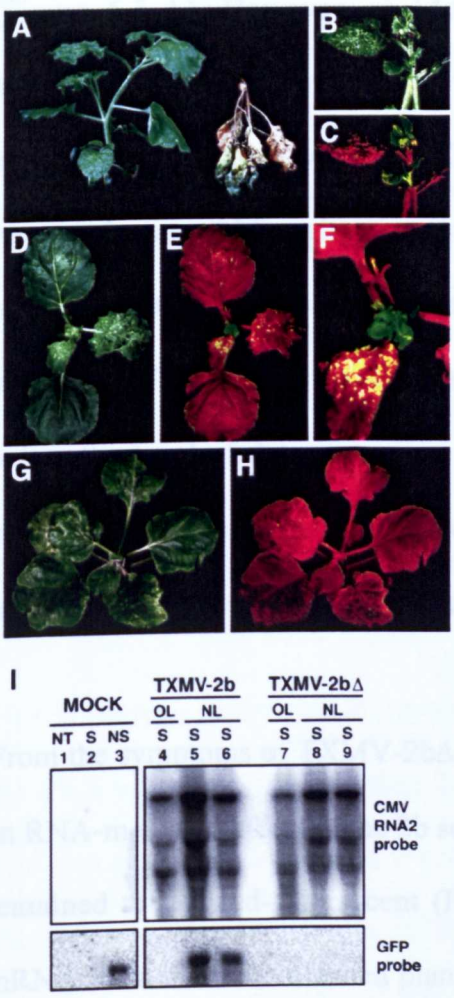

Figure 6.5. Suppression of PTGS by the 2b protein of CMV (**A**) Non-transformed *N. benthamiana* plants inoculated with TXMV-2bΔ (left) and TXMV-2b (right). The photograph was taken at 21 days post inoculation and both plants were the same age when inoculated. (**B**) GFP-silenced *N. benthamiana* line 16c showing symptoms of TXMV-2b infection at 21d post inoculation. (**C**) The same plant shown under UV illumination revealing the PTGS of GFP persisted in symptomatic leaves that had emerged from meristems before systemic infection but that it is suppressed in the post emergence leaves. (**D**) and (**E**) are aerial views of the plant shown in (B) under white light and UV illumination. (**F**) shows the apical zone from the image in (E). (**G, H**) GFP-silenced *N. benthamiana* line 16c showing symptoms of TXMV-2bΔ under white light (G) and UV illumination (H). (**I**) Northern analysis of RNA extracted at 15d post inoculation from either NT, non-silenced (NS) or silenced (S) plants inoculated with TXMV-2b and TXMV-2bΔ. The RNA samples were taken either from old leaves (OL) that had emerged before systemic virus infection or from new leaves (NL) emerging after the virus had spread systemically. 5 µg of total RNA per sample were fractionated by electrophoresis on a 0.9% (w/v) agarose-formaldehyde gel, blotted onto a nylon membrane and hybridised with probes specific for RNA2 of CMV or for GFP RNA as indicated.

inoculated a PVX vector expressing the CMV 2b protein (TXMV-2b; Figure 6.1) to GFP-silenced *N. benthamiana*. We also infected GFP-silenced plants with TXYMV-2bΔ in which a single nucleotide substitution (U to A) converted the fourth codon (UUG) of the 2b open

reading frame to a stop codon (UAG) (Figure 6.1). TXMV-2b and TXMV-2bΔ were kindly provided by Shou Wei Ding. By three weeks post-inoculation TXMV-2b produced symptoms that were much more severe than those produced by wild type PVX or by TXMV-2bΔ (Figure 6.5). Instead of the normal mild mosaic symptoms of PVX, TXMV-2b induced necrosis on the systemically-infected leaves and stem, leading to death of the plants (Figure 6.5-A). However, at 14d post-inoculation, before the systemic necrosis had developed, the newly emerging leaves of TXMV-2b-infected plants were green fluorescent under UV illumination. As in the CMV-infected plants, the leaves that had emerged prior to virus infection, although symptomatic, remained red fluorescent (Figure 6.5-F). The phenotype of TXMV-2b-infected plants was associated with corresponding changes in the levels of GFP RNA. In the older, red fluorescent leaves, the level of GFP RNA remained below the level of detection as in mock-infected leaves (Figure 6.5-I, tracks 2 and 4). In contrast, in the new green fluorescent leaves, the GFP RNA had increased to the levels of non-silenced plants (Figure 6.5-I, tracks 3, 5 and 6).

From the symptoms of TXMV-2bΔ we ruled out that the suppression of GFP silencing was an RNA-mediated effect of the 2b sequence. GFP silenced plants infected with this construct remained totally red-fluorescent (Figure 6.5-G and H) and contained low levels of GFP mRNA as in the GFP-silenced plants that had been mock inoculated (Figure 6.5-I, tracks 2 and 7-9). We also ruled out that the suppression of GFP silencing was due to a non-specific enhancement of transgene expression by the 2b protein by showing that TXMV-2b had no effect on GFP RNA or the green fluorescence of the non-silenced GFP lines (data not shown). Therefore, from the similarity of the CMV and TXMV-2b effects, we conclude that

the 2b protein is the major suppressor of gene silencing encoded in the CMV genome.

6.7. Discussion

In this study, two viral suppressors of PTGS encoded by CMV and PVY were identified. These findings support the previous suggestion that PTGS in plants is a natural protection mechanism against viruses (Chapter 3, (Pruss et al., 1997; Ratcliff et al., 1999)). Furthermore, by implicating PTGS in resistance against two distinct types of RNA virus, our findings indicate that this mechanism may have general significance in viral pathogenesis of plants.

6.7.1. Suppressors at different stages of PTGS

The two viral suppressors of PTGS in plants identified in this work are HCPro encoded by the PVY genome and the 2b protein encoded by the RNA 2 of CMV. Expression of either of these proteins from a PVX vector suppressed PTGS of a GFP transgene. This effect was clearly protein- rather than RNA-mediated because there was no suppression of PTGS when the PVX vector carried modified forms of the HCPro and the 2b coding sequences. We can rule out that suppression of PTGS was due to a non-specific stimulation of the GFP transgene expression because GFP fluorescence and RNA levels remained unaffected in a non-silenced line infected with PVY, TXYHC or CMV. We can also rule out that the results obtained are an artefact associated with a virus vector because P1-HCPro of tobacco etch virus expressed in transgenic plants is also a suppressor of PTGS (Anandalakshmi et al., 1998).

In a previous analysis of GFP silencing, we showed that initiation, systemic spread of a silencing signal and maintenance are separate stages of the PTGS mechanism (Chapter 5). Here, from the GFP phenotype in virus-infected plants we have shown that these stages are differentially targeted by the two viral suppressors. In plants infected with PVY or TXYHC there was suppression of PTGS in all of the symptomatic tissues, including the older leaves that would have received the systemic signal and initiated PTGS before the viruses had established infection. Based on these observations, we conclude that HCPro must be blocking at least the maintenance stage of PTGS, although our data do not rule out that initiation and systemic spread are also affected. The extent of suppression of PTGS was correlated with the level of PVY or TXYHC RNA which suggests that high levels of the HCPro are required for suppression of PTGS.

Suppression of PTGS in CMV- or PVX-2b-infected plants was unlike the HCPro-mediated effect, because the only affected leaves were those that emerged from the growing points after the viruses had spread systemically. From this pattern, we conclude that the 2b protein affects the cells in, or near to, the growing points of the plants. The growing points do not normally exhibit PTGS, even when there is extensive silencing in the rest of the plant (chapter 5). Therefore, it is possible that the 2b protein prevents entry of the gene silencing signal to the cells emerging from the growing points. Alternatively, it could be that the signal enters these cells but that the 2b protein prevents initiation of the PTGS mechanism.

6.7.2. Viral symptom determinants and suppressors of gene silencing

From the results presented here and elsewhere, it is shown that the HCPro and the 2b proteins have several activities. First, these proteins are pathogenicity factors of the respective viruses (Cronin et al., 1995; Ding et al., 1995). They also enhance the accumulation and symptoms of PVX when they are expressed from PVX vectors (Pruss et al., 1997) and, as shown here, they suppress PTGS. In addition, in protoplasts from transgenic plants expressing HCPro, there is enhanced accumulation of PVX, TMV or CMV (Pruss et al., 1997).

Although it remains formally possible that these are unrelated effects of the two proteins, a more likely explanation is that they are all associated with the suppression of a PTGS-like resistance mechanism. From the findings that PVY, CMV and the various PVX constructs are all affected by this mechanism, we propose that it is activated or given specificity by different types of RNA virus. We envisage that this mechanism could target degradation of RNA species that are similar to the inoculated virus. The effect of the mechanism would be to restrict viral RNA accumulation in infected cells. Additionally, as shown in transgene-induced PTGS we consider it possible that there is a sequence-specific signal molecule that spreads away from the cells in which the process is initiated (Chapter 5). The potential to produce and transmit a signal out of the initially-infected cell could mean that this PTGS-like resistance mechanism has the ability to activate viral RNA degradation in non-infected cells located beyond the front of infection. Consequently, the PTGS-like mechanism could be responsible for suppression of virus movement as well as restriction of viral accumulation in infected cells.

It is unlikely that a PTGS-like resistance mechanism is specific to the potex, cucumo- and potyviral groups. These groups represent extremes in the evolution of positive strand RNA viruses having either monopartite or multipartite genome organisation and similarity to either the picorna- or sindbis virus groups (Matthews, 1991). Therefore, the shared property of these viruses as activators of the PTGS-like resistance mechanism, is probably common to most groups of positive strand RNA viruses in plants. Moreover, if a PTGS-like mechanism is implicated in virus resistance, it is expected that other virus-encoded proteins will function as suppressors of gene silencing. From the results presented here, we predict that many viral proteins that were originally identified as pathogenicity determinants, like the 2b protein or HCPro, will eventually be identified as suppressors of PTGS.

The lack of an effect of PVX on the GFP silenced plants suggests that this virus does not apparently produce a suppressor of PTGS. However, the increase in symptoms and virus accumulation in plants infected with TXYHC and PTXMV-2b implies that PVX does activate the PTGS-like resistance in natural infections. There are two ways to reconcile these observations. Either PVX encodes a suppressor that targets a step of the PTGS mechanism that was not evidenced through the silencing reversal assay used here. Alternatively, PVX may have the potential to partially evade rather than to suppress the PTGS-like resistance mechanism. Evasion might involve very rapid replication and spread of the virus so that the resistance mechanism is out-competed. Another possibility is that PVX might be compartmentalised, so that it avoids the PTGS-like mechanism.

6.7.3 Viral adaptations to gene silencing

The general principles of suppression and evasion of the PTGS-like resistance mechanism will probably apply to many plant-virus interactions. Evasive or strongly suppressive viruses will be highly pathogenic, whereas the others will be weak or non-pathogens. The likely central role of this mechanism in plant virus interactions means that there will be strong selective pressures on the virus to evade or suppress the mechanism. Similarly, there will be corresponding selective pressures on the plant side to ensure that the mechanism is effective against many viruses. These selective pressures will probably cause a high level of variation in both the plant and the viral genes involved in the mechanism. Clearly, identification of other viral suppressors and further investigation of this proposed adaptive mechanism will certainly provide support for a generalised virus resistance mechanism in plants. In addition, it is hoped that identification of viral suppressors of PTGS will provide an experimental handle for the characterisation of host components involved in PTGS.

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CHAPTER 7

Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants.

7.1. Abstract

In transgenic and non-transgenic plants, viruses are both initiators and targets of a defence mechanism that is similar to post-transcriptional gene silencing (PTGS). We have shown that potyviruses and cucumoviruses encode pathogenicity determinants that suppress this defence mechanism. Here, we test diverse virus types for the ability to suppress PTGS. *Nicotiana benthamiana* exhibiting PTGS of a green fluorescent protein (GFP) transgene were infected with a range of unrelated viruses and various potato virus X (PVX) vectors producing viral pathogenicity factors. Upon infection, suppression of PTGS was assessed *in planta* through reactivation of green fluorescence, and confirmed by molecular analysis. These experiments led to the identification of three novel suppressors of PTGS and showed that suppression of PTGS is widely used as a counter-defence strategy by DNA and RNA viruses. However, the spatial pattern and degree of suppression varied extensively between viruses. At one extreme there are viruses that suppress in all tissues of all infected leaves, whereas others are able to suppress only in the veins of new emerging leaves. This variation existed even between related members of the potexvirus group. Collectively, these results suggest that virus-encoded suppressors of gene silencing have distinct modes of action, are targeted against distinct components

of the host gene silencing machinery and that there is dynamic evolution of the host and viral components associated with the gene silencing mechanism.

7.2. Introduction

In chapter 6, I have described the identification of 2 viral-encoded suppressors of gene silencing. Although 2b and HcPro act at different stages of the PTGS mechanism and are dissimilar at the protein sequence level, they are both pathogenicity determinants of their respective viruses. By extrapolation, it was predicted that many viral pathogenicity determinants would be identified as suppressors of gene silencing and that, more generally, many viruses would have the ability to suppress PTGS. Here, I test this idea by infecting *N.benthamiana* plants exhibiting PTGS of a GFP transgene with a range of viruses. Plants were also infected with PVX vectors expressing previously identified viral pathogenicity determinants. As reasoned in chapter 6, if these wild type and recombinant viruses produced suppressors of a PTGS-like resistance mechanism, we expected that they would interfere with PTGS of GFP. The outcome of these experiments was consistent with the initial prediction and revealed that suppression of gene silencing is a widespread strategy among plant viruses. This study led to the identification of three novel viral suppressors of PTGS and revealed an intriguing phenotype of silencing suppression that operates in the vicinity of the veins.

7.3. Suppression of gene silencing by diverse plant viruses

The test for silencing suppression used in the following experiments was as described in chapter 6. Systemic silencing in *N.benthamiana* line 16c was induced by infiltration of lower leaves of transgenic seedlings with the epiGFP strain of *Agrobacterium tumefaciens* (Chapter 5, section 5.3.1). By 20 days post-infiltration, silencing of the GFP was extensive in all vegetative tissues of the plants, and, consequently, they appeared uniformly red under UV illumination. At this stage, there was no PTGS in the growing points of the plant and silencing was maintained by being constantly initiated in non silenced cells located near or in the meristems (Chapter 5). These silenced plants were then infected with a range of plant viruses and, when systemic symptoms were observed, the extent of green fluorescence was assessed under UV illumination. In addition, Northern analysis was performed to assess the level of GFP mRNAs in infected tissues. Our findings were that many, but not all of the viruses tested, suppressed gene silencing in *N. benthamiana* (Table 7.1). With several viruses, suppression occurred in old leaves (OL) that had emerged before the virus had spread, as well as in new emerging leaves (NL). This was reminiscent of the pattern of silencing suppression previously described for PVY. In contrast, tomato bushy stunt virus (TBSV) only suppressed gene silencing in new emerging tissues, as was reported for CMV in the previous chapter. Foxtail mosaic virus (FoMV), alfalfa mosaic virus (AMV) or tobacco black ring virus (TBRV) were like PVX in that they were fully infectious but did not have any effect on GFP silencing. From the diversity of viruses tested in this analysis, we conclude that PTGS suppression is a property of many plant viruses. However, because the spatial pattern and degree of

suppression varied extensively between viruses, it was likely that different mechanisms would be involved.

Virus group	Virus	Suppression of PTGS	Old leaves/ New leaves	Whole leaf / Vein centric	Protein	Other known functions[b]
Alfamovirus	ALMV	0/9	-	-	-	-
Comovirus	CpMV	5/6	OL and NL	Vein centric	?	-
Cucumovirus	CMV	20/20	NL only	Whole leaf	2b	Host-specific long distance movement
Geminivirus	ACMV	6/6	OL and NL	Whole leaf	AC2	Virion sense gene expression transactivator
Nepovirus	TBRV	0/6	-	-	-	-
Potexvirus	PVX	0/9	-	-	-	-
	FoMV	0/9	-	-	-	-
	NMV	8/9	OL and NL	Whole leaf	?	-
	NVX	7/9	OL and NL	Whole leaf	?	-
	VMV	7/9	OL and NL	Whole leaf	?	-
Potyvirus	PVY/ TEV	10/10	OL and NL	Whole leaf	HcPro	Genome amplification Viral synergism Long distance movement Polyprotein processing Aphid transmission
Sobemovirus	RYMV	-[a]	-[a]	-[a]	P1	Virus accumulation Long distance movement
Tobamovirus	TMV	4/6	OL & NL	Vein centric	?	-
Tobravirus	TRV	7/9	OL & NL	Whole leaf	?	-
Tombusvirus	TBSV	7/9	NL only	Vein centric	19kDa	Host-specific spread and symptom determinant

Table 7.1. Suppression of PTGS of GFP mRNA caused by various plant viruses. PTGS of the GFP mRNA was induced in transgenic *N.benthamiana* by *Agrobacterium* infiltration, as described (Chapter 5). Following systemic infection, suppression of gene silencing was assessed under UV illumination throughout time and confirmed by RNA gel blot analysis. RNA Samples were taken from either old leaves that had emerged before the virus had spread systemically (OL) or from new leaves emerging after virus infection (NL). The total number of plants tested is indicated as well as the phenotype of suppression in leaves (affecting whole tissues or vein centric). Viruses were tested in duplicate independent experiments during the summer and the winter.

[a] RYMV is not infectious in *N.benthamiana*. The P1 protein has been identified as a PTGS suppressor by expression from the PVX vector.

7.4. The geminivirus-encoded AC2 protein is a suppressor of gene silencing

As shown in Table 7.1, infection of African cassava mosaic geminivirus (ACMV) led to suppression of GFP silencing at about three weeks post-inoculation in both fully expanded and new emerging infected tissues (Figure 7.1-B). Correspondingly, Northern

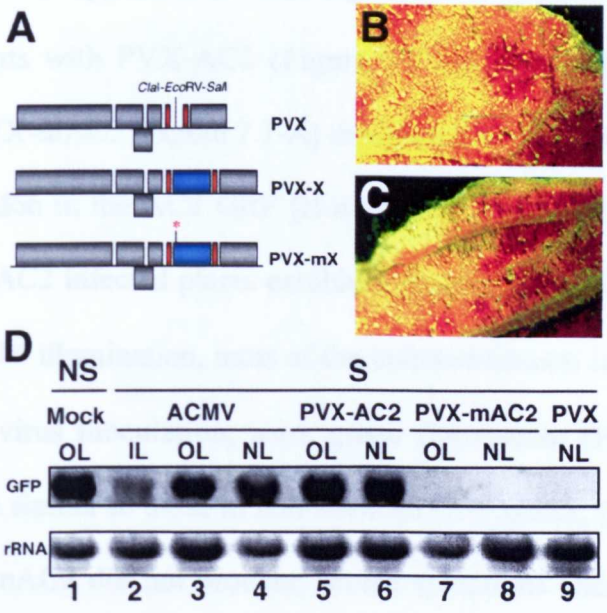

Figure 7.1. Suppression of PTGS by ACMV and PVX-AC2. (**A**) Schematic representation of the PVX vector used to express various pathogenicity determinants tested in this study (referred to as "X"). Individual sequences were inserted into the P2C2S PVX vector using the *Cla*I-*Eco*RV-*Sal*I multiple cloning site (see also chapter 6), leading to "PVX-X". Mutant versions of all pathogenicity determinants, referred to as "mX", were also used in this study (mutation indicated by a red asterisk). (**B**) Close-up of an ACMV-infected leaf from a GFP-silenced *N.benthamiana* (**C**) Close-up of a PVX-AC2 infected leaf from a GFP-silenced *N.benthamiana*. Photos from (B) and (C) were taken under UV illumination from a dissecting microscope at 15 DPI. (**D**) Northern analysis of RNA extracted at 20 DPI from either mock infected, non-silenced (NS) or silenced (S) *N.benthamiana* infected with ACMV, PVX-AC2, PVX-mAC2 or PVX. RNA samples were taken either from inoculated leaves (IL), old leaves that had emerged before the virus had spread systemically (OL), or from new leaves emerging after virus infection (NL). Equal amounts of each RNA sample (10μg) were assayed by RNA gel blotting, using a ^{32}P-labeled GFP cDNA as probe. Ethidium bromide staining of ribosomal RNA (rRNA) shows equal loading of the samples.

analysis revealed that GFP mRNA levels were high in both types of tissues, and that suppression also occurred in inoculated leaves, although to a lower extent (Figure 7.1-D, tracks 1-4). Therefore, these results were consistent with a suppressor of PTGS encoded in the ACMV genome. To identify this putative suppressor, we exploited previous findings that a PVX vector expressing the AC2 protein (PVX-AC2) produced necrotic symptoms that were much more severe than those of wild type PVX, suggesting that AC2 suppressed a host defence mechanism (Hong et al., 1997). From the above results, it was likely that AC2 was a suppressor of silencing. The test of this hypothesis was to infect GFP-silenced plants with PVX-AC2 (Figure 7.1-A). As a control, plants were also inoculated with PVX-mAC2 (Figure 7.1-A) in which a single point mutation introduces a premature stop codon in the AC2 ORF (Hong et al., 1997). At about two weeks post-inoculation, PVX-AC2 infected plants exhibited severe symptoms, as reported (Hong et al., 1997). Under UV illumination, most of the infected tissues, including leaves that had emerged prior to virus inoculation, were green fluorescent (Figure 7.1-C) and GFP mRNA levels were similar to those in non-silenced GFP plants, (Figure 1D, tracks 5, 6). In contrast, PVX-mAC2 did not produce severe symptoms and did not suppress GFP silencing (Figure 7.1-D, tracks 7-8). From these results, we conclude that the AC2 protein encoded in the ACMV genome is a suppressor of maintenance of PTGS in *N. benthamiana.*

7.5. Vein-associated suppression of silencing by the 19K protein of TBSV

N. benthamiana infected with TBSV showed reversion of PTGS at about 3 weeks post-inoculation, when symptoms were fully systemic (Table 7.1). As in CMV infected plants the restoration of green fluorescence occurred only in new emerging infected leaves. However, this suppression of silencing was weaker than with CMV, so that the green fluorescence was barely detectable under UV illumination from a hand-held lamp. Also unlike CMV, TBSV only suppressed PTGS in and around the veins (Figure 7.2-A). Vein-

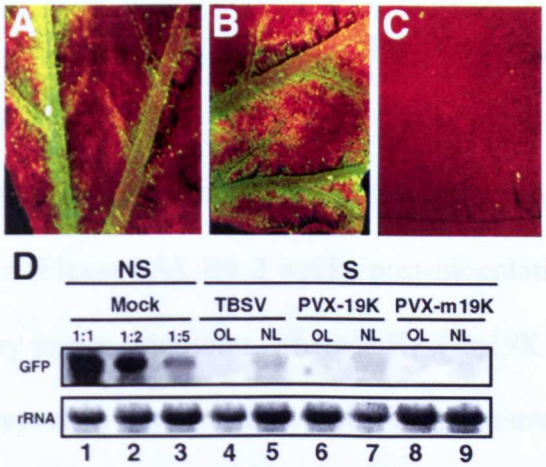

Figure 7.2. Vein-centred suppression of PTGS caused by TBSV and PVX-19K. (**A**) Close-up of a TBSV-infected leaf from a GFP-silenced *N.benthamiana* (**B**) Close-up of a PVX-19K infected leaf from a GFP-silenced *N.benthamiana.* (**C**) Close-up of a PVX-m19K infected leaf from a GFP-silenced *N.benthamiana.* Photographs from (A), (B) and (C) were taken under UV illumination from a dissecting microscope at 20 DPI. (**D**) Northern analysis of RNA extracted at 20 DPI from silenced (S) *N.benthamiana* infected with PVX-19K or PVX-m19K. RNA samples were taken either from old leaves (OL) or from new emerging leaves (NL). Equal amounts of each RNA sample (15µg) were assayed by RNA gel blotting, using a ^{32}P-labeled GFP cDNA as probe. Lanes 1-3 show a dilution series of GFP RNAs from a non silenced (NS) plant into total RNA from a non-transformed (NT) plant. GFP RNA was diluted to a half (1:2) or to a fifth (1:5) of the reference sample (1:1). Ethidium bromide staining of ribosomal RNA (rRNA) shows equal loading of the samples.

centred reversion of GFP was more evident when detached, new emerging leaves were observed under a dissecting microscope (Figure 7.2-A). Northern analysis showed that GFP RNAs were more abundant in the new leaves of the infected plants than in old

leaves or in mock-inoculated, non-silenced plants. However, the GFP RNA in the new leaves was less than 20% of the level in mock inoculated plants (Figure 7.2-D, tracks 4,5). It has been reported that the 19K protein of TBSV is a pathogenicity determinant. For example, a PVX vector expressing the 19K protein (pHS142), referred to here as PVX-19K (Figure 7.1-A), induced severe symptoms on *N. benthamiana* (Scholthof et al., 1995). In addition, inactivation of the 19K protein in TBSV had an attenuating effect on the lethal apical necrotic symptom phenotype that is usually elicited in plants by TBSV (Scholthof et al., 1995). Collectively these data indicate that the TBSV 19K protein possesses attributes of a suppressor of gene silencing. To test this hypothesis, silenced GFP plants were inoculated with PVX-19K (Figure 7.1-A). As a control, plants were also inoculated with pHS160 (referred to here as PVX-m19K) carrying a non-translatable form of the 19K protein (Figure 1A). By 2 weeks post-inoculation, plants infected with PVX-19K exhibited very severe symptoms, whereas PVX-m19K infected plants had mild mosaic symptoms, as reported, Suppression of silencing occurred in PVX-19K-infected plants but was only manifested in new emerging tissues and was most pronounced in the veins (Figure 7.2-B). However, symptoms of PVX-19K were visible on all areas of the leaves (not shown). Similar tissues infected with PVX-m19K remained uniformly red-fluorescent (Figure 7.2-C). Northern analysis of RNA extracted from new emerging, infected leaves showed that only low levels of GFP RNAs could be detected in PVX-19K-infected tissues (Figure 7.2-D, track 6, 7) and that GFP RNAs were below the level of detection in PVX-m19K-infected tissues (Figure 7.2-D, track 8, 9). Taken together, these results suggest that the 19K protein of TBSV is a suppressor of PTGS in *N.benthamiana* that operates in the vicinity of the vein tissues of new-emerging leaves.

7.6. Other examples in which suppression of PTGS occurs preferentially in or near the veins

As part of our survey, we investigated the effect of tobacco mosaic virus (TMV) and cowpea mosaic virus (CPMV), type members of the tobamovirus and comovirus groups, respectively (Matthews, 1991). Inoculation of the corresponding viruses onto GFP silenced plants led to suppression of gene silencing that affected both new emerging and already expanded silenced tissues, thus indicating that maintenance of PTGS was

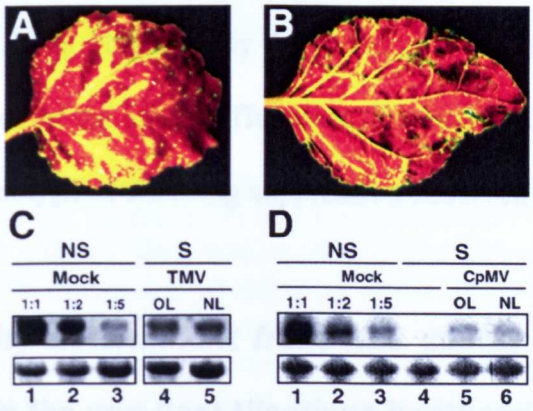

Figure 7.3. Suppression of PTGS caused by TMV and CPMV occurs preferentially in the vicinity of the veins. (**A**) Close-up of a TMV-infected leaf from a GFP-silenced *N.benthamiana* (**B**) Close-up of a CPMV infected leaf from a GFP-silenced *N.benthamiana.* Photographs from (A) and (B) were taken under UV illumination from a hand-held lamp at 20 DPI. (**C**) Northern analysis of RNA extracted at 20 DPI from silenced (S) *N.benthamiana* infected with TMV. RNA samples were taken either from old leaves (OL) or from new emerging leaves (NL). Equal amounts of each RNA sample (15µg) were assayed by RNA gel blotting, using a ^{32}P-labeled GFP cDNA as probe. Samples were separated on the same agarose gel and blotted on the same filter that was used in FIG 2, thus allowing the use of the same GFP RNA dilution series as a reference. (**D**) Northern analysis of RNA extracted at 20 DPI from silenced (S) *N.benthamiana* infected with CPMV. Equal amounts of each RNA sample (15µg) were assayed by RNA gel blotting, using a ^{32}P-labeled GFP cDNA as probe. Mock control lanes 1-3 were prepared as in Figure 2. Ethidium bromide staining of ribosomal RNA (rRNA) shows equal loading of the samples.

alleviated (Table 7.1, Figure 3A and 3B). However, as shown previously for TBSV and PVX-19K, suppression was mostly manifested near, or in the veins, with most tissues of

the lamina remaining silenced (i.e. red fluorescent), although symptoms of the respective viruses were observed on the whole leaf lamina (data not shown). This phenotype did not change throughout time, even when infected leaves were fully expanded and completely infected. With both viruses, green fluorescence in the vicinity of the veins was very strong and this effect was clearly apparent under UV illumination from a hand-held lamp. Northern analysis of RNAs extracted from infected leaves showed that GFP RNA accumulation was restored in those tissues, but at a low level, when compared to the abundance of GFP RNA extracted from similar tissues of non-silenced, non-infected plants (Figure 3C, 3D). This was probably due to dilution of the vein tissue into the most abundant silenced tissues of the lamina. Therefore, this molecular analysis was consistent with the particular phenotype of silencing suppression observed under UV illumination.

7.7. A pathogenicity determinant from rice yellow mottle virus (RYMV) suppresses PTGS in the non-host Nicotiana benthamiana species.

RYMV is a sobemovirus exhibiting a very narrow host range. It only systemically infects monocotyledonous species belonging to the Oryzae, Phalaridae and Eragrostidae tribes (Bakker, 1974). Recent studies have characterised the P1 protein of RYMV as an important pathogenicity determinant in rice (Bonneau et al., 1998). To test if it would suppress gene silencing in a RYMV non-host species, the P1 ORF was introduced into the PVX vector and GFP-silenced *N. benthamiana* were infected with the resulting recombinant virus (PVX-P1, Figure 7.1-A). As a control, a PVX vector carrying a non-translatable form of P1 (PVX-mP1, Figure 1A) was also inoculated. At about two weeks

post-inoculation, tissues infected with PVX-P1 exhibited severe chlorosis and white necrosis (Figure 7.4-D).

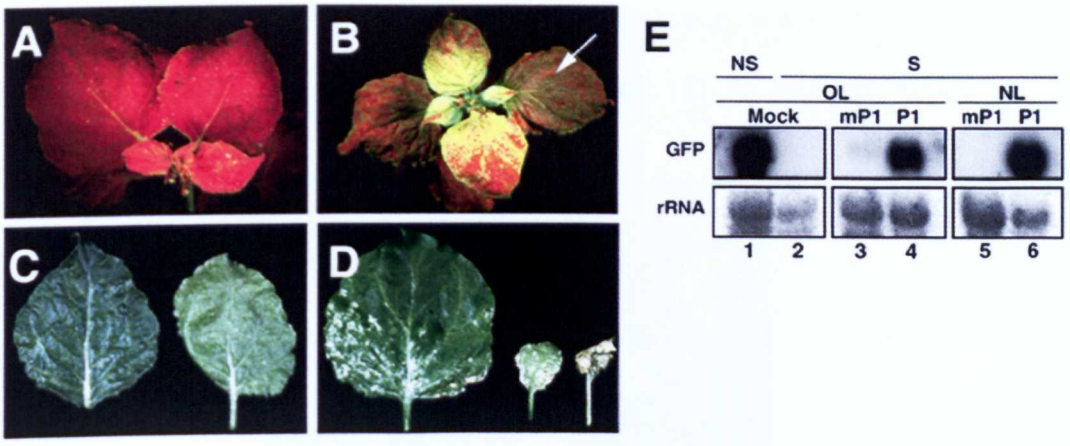

Figure 7.4. Severe symptoms and suppression of PTGS caused by PVX expressing the RYMV P1 protein. (**A**) UV illumination of a GFP-silenced *N.benthamiana* infected with PVX-mP1 at 14 DPI. (**B**) UV illumination of a GFP-silenced *N.benthamiana* infected with PVX-P1 at 14 DPI. Reversion of silencing occurs in both new emerging tissues as well as in old leaves (indicated by an arrow). (**C**) Mild mosaic symptoms caused by PVX-mP1 at 14 DPI. (**D**) Severe necrotic symptoms caused by PVX-P1 at 14 DPI. (**E**) Northern analysis of RNA extracted at 14 DPI from either mock-infected, non-silenced (NS) or silenced (S) *N.benthamiana* infected with PVX-P1 or PVX-mP1. RNA samples were taken either from old leaves (OL) or new emerging leaves (NL). Equal amounts of each RNA sample (10μg) were assayed by RNA gel blotting, using a ^{32}P-labeled GFP cDNA as probe. Ethidium bromide staining of ribosomal RNA (rRNA) shows equal loading of the samples

Under UV illumination, these tissues, including leaves that had emerged prior to virus inoculation, appeared green fluorescent (Figure 7.4-B). Accordingly, in young infected tissues, GFP mRNA levels were similar to those in non-silenced GFP plants (Figure 7.4-E, track 6). GFP mRNAs could also be detected in infected leaves that had emerged prior to virus inoculation, although to a lower extent (Figure 7.4-E, track 4). In contrast, neither severe symptoms nor reversion of GFP silencing was caused by PVX-mP1-infection (Figure 7.4-A, 4C, 4E tracks 3 and 5). From this data, we conclude that the P1 protein of

RYMV is a suppressor of maintenance of PTGS in *N. benthamiana*, although it is encoded in the genome of a virus that is not infectious on *Nicotiana* species.

7.8. Strong variations in the ability to suppress PTGS in N. benthamiana are observed between highly related members of the potexvirus group.

PVX and FoMV, both members of the potexvirus group, had no effect on PTGS of GFP in *N.benthamiana* (Table 7.1, Figure 7.5-C, track 10). In contrast, infection with other

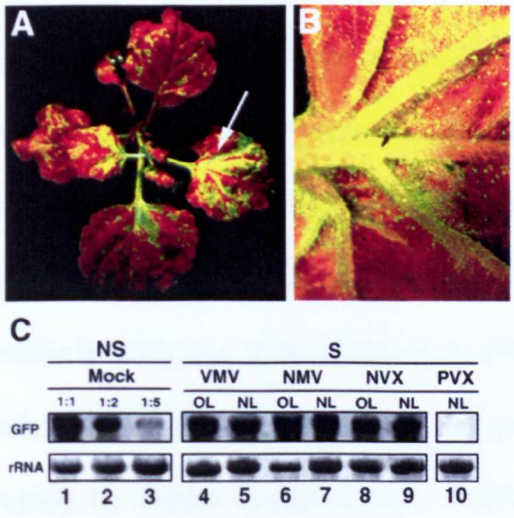

Figure 7.5. Effect of various potexviruses on PTGS of GFP. (**A**) UV illumination of a GFP-silenced *N.benthamiana* infected with NMV at 20 DPI. Reversion of silencing occurs in both new emerging tissues as well as in old leaves (indicated by an arrow). (**B**) Close-up of a NVX infected leaf from a GFP-silenced *N.benthamiana*. This photograph was taken under UV illumination from a dissecting microscope at 20 DPI. (**C**) Northern analysis of RNA extracted at 20 DPI from silenced (S) *N.benthamiana* infected with either VMV, NMV, NVX or PVX. RNA samples were taken either from old leaves (OL) or from new emerging leaves (NL). Equal amounts of each RNA sample (15µg) were assayed by RNA gel blotting, using a ^{32}P-labeled GFP cDNA as probe. Samples were separated on the same agarose gel and blotted on the same filter that was used in FIG 2, thus allowing the use of the same GFP RNA dilution series as a reference. Ethidium bromide staining of ribosomal RNA (rRNA) shows equal loading of the samples.

potexviruses, narcissus mosaic virus (NMV), nandina virus X (NVX) and viola mosaic virus (VMV), led to suppression of gene silencing in *N. benthamiana*. This suppression was manifested in leaves that were expanded prior to inoculation as well as in young developing tissues (Figure 7.5-A, 5B, 5C). The suppression was as strong as with Hc-pro,

2b and AC2 and the levels of GFP mRNA in infected tissues were similar to those in mock inoculated, non-silenced plants (Figure 7.5-C).

The inocula of these related viruses had been quantified using the local lesion host *Chenopodium amaranticolor* and diluted, so that they would be comparable to a PVX inoculum used as an internal reference (40 lesions per leaf). Following infection, we confirmed that these viruses gave similar types of symptoms. Thus, the variation in the suppressor of silencing activity reflected intrinsic properties of the viruses rather than the degree of infection. Surprisingly the variable suppressor activity did not correlate with the nucleotide sequence similarity of these viruses. PVX and FoMV, which did not suppress silencing, are only distant relatives. In contrast, NVX and VMV, which produced strong suppressors, are respectively 93% and 97% identical to PVX at the nucleotide level, based on sequence analysis of a region spanning the coat protein and the 3 movement proteins (kindly provided by Abdel Bendhamane). NMV, which also produced a suppressor, is only a distant relative of PVX. Therefore, there is extreme variation in the ability to suppress PTGS in closely related members of a single virus group.

7.9. Discussion

7.9.1. Suppression of PTGS as a general strategy

We previously predicted that many viruses would encode proteins that are suppressors of an silencing mechanism and that these proteins would also suppress PTGS. The likely candidate suppressors were viral proteins that, like the 2b protein or HcPro, were originally characterised as pathogenicity determinants. Consistent with this hypothesis, the ACMV AC2, the RYMV P1 and the TBSV 19K pathogenicity factors all suppress

PTGS of a GFP transgene. It is therefore likely that the activity of these proteins in pathogenicity of the encoding virus is associated with suppression of silencing. The ability of these proteins to enhance symptoms of PVX vectors is most likely explained in the same way. The finding that a DNA geminivirus, ACMV, encodes a suppressor was not surprising because other geminiviruses are known to induce PTGS in transgenic and non-transgenic plants (Atkinson et al., 1998; Kjemtrup et al., 1998).

Each virus produced a characteristic pattern of silencing suppression. Some, like potyviruses, suppressed in young and old leaves. Others were like CMV and affected only young leaves. There was also variation in the tissue specificity with ACMV, VMV, NMV, NVX and PVX-P1 affecting all tissues whereas TBSV, TMV and CPMV specifically suppressed silencing in tissues that were in or close to the veins. It is umlikely that these differences reflect the tissue tropism of these viruses. Similar patterns were reproduced when various suppressors were expressed from a PVX vector that has been shown to express foreign proteins uniformly throughout infected leaves. A more likely explanation depends jointly on the mode of action of the suppressor and the component of the gene silencing mechanism that is targeted. For example, if a suppressor can degrade a component required for maintenance of gene silencing, it will have an effect in both new and old leaves. However, if the suppressor blocks synthesis or activation of a component required for silencing, the suppression would be restricted to new emerging leaves where silencing would be established in the presence of the viral suppressor. In old leaves, the component would have been formed in the absence of the suppressor and, consequently, would be unaffected when the virus would infect the plant.

The suppression of silencing in or near the veins, for example with the 19K protein of TBSV, could indicate that this protein is only stable or expressed in the veins, or that it is targeted against a component of the PTGS mechanism that is qualitatively or quantitatively different between vascular and non vascular tissue. Alternatively, the suppressor could be targeted against the systemic signal of PTGS. It was shown that this signal is phloem-transmitted and that, in recipient leaves, it is primarily located in and near the veins. Of these alternative explanations for suppression of silencing in veins, those involving vein-specific components or stability of the suppressors are unlikely because, in all cases, PTGS suppression extended into cells outside the vascular bundle and appeared to reflect movement of the signal rather than a precisely vein-specific silencing process. For this reason, it is possible that the suppressors of TMV, CPMV and TBSV are targeted against the systemic signal of silencing and may therefore represent a viral adaptation to systemic silencing.

Although TMV, TBSV and CPMV are only able to suppress PTGS in or near the veins, they are nevertheless able to accumulate at a high level throughout the infected leaf. It is likely, therefore, that these viruses have secondary strategies for counteracting the effects of silencing. These strategies may involve evasion, so that the process is not activated, or escape from the antiviral mechanism. Luteoviruses, which are typically restricted to the phloem (Matthews, 1991) may provide an interesting example of viruses that are unable to either suppress, evade or escape from the effect of silencing outside the veins. Consistent with this idea, it has been reported that the level of potato leafroll luteovirus

(PLRV) increased up to twelve fold in *Nicotiana* species that were co-infected with NMV, tobacco rattle virus (TRV) or PVY (Barker, 1989). It now seems likely that this increase was due, at least in part, to the ability of PLRV to spread beyond the veins due to suppression of silencing in the double-infected plants. Here, it is shown that NMV, TRV and PVY are all able to suppress maintenance of PTGS in *N. benthamiana* (Table 7.1). In contrast, co-infection with AMV or TBRV that are unable to suppress PTGS (Table 7.1) did not alter PLRV concentration in leaves (Barker, 1989).

7.9.2. Gene silencing activation/suppression as a co-evolutive mechanism?

It is striking that the viral suppressors of silencing are so diverse. So far, we have been unable to identify any common structural features in these proteins and we conclude that the suppressor function has evolved independently several times as a strategy to counteract the effects of silencing. In some instances, it is conceivable that some suppressors have converged towards the same function and thus, are targeted against similar components of the silencing machinery. For example, the RYMV P1 protein shares striking functional similarities with the potyviral-encoded HcPro protein. First, when produced from the PVX vector, both proteins are suppressors of maintenance of PTGS in *N.benthamiana.* In addition, both proteins are required for efficient accumulation of viral RNAs in protoplasts and long distance movement in their respective host (Bonneau et al., 1998; Kasschau et al., 1997).

Because silencing is likely to have a central role in plant-virus interactions, one can also anticipate that there will be a dynamic evolution of plant components required for the

mechanism and, accordingly, of the virus-encoded components necessary to overcome it. The poty- and potexvirus groups probably represent different stages in this dynamic evolution. In the potyvirus group, the HcPro of TEV, PVY and pea seedborne mosaic virus (data not shown) are suppressors of GFP silencing in *Nicotiana* species. In these viruses, the suppressor seems to be an evolutionarily conserved function and its corresponding target is likely also to be conserved in different plants. In contrast, the potexvirus strategy for counteracting PTGS is apparently in a state of evolutionary flux. Presumably, PVX and FoMV, as opposed to VMV and NVX, do not have a functional suppressor of silencing in *N.benthamiana* and, on that host, must use alternative strategies to escape or evade the mechanism, as proposed above. However, it might be expected that on other hosts, PVX and FoMV would produce functional suppressors and conversely VMV and NVX would not. The test of this co-evolution hypothesis would require a suitable set of host plant species exhibiting PTGS, rather than the single GFP *N. benthamiana* line used here.

In due course, it may transpire that the balance between silencing activation and suppression will strongly influence virus-host interactions. For example, if a virus cannot suppress, evade or escape the effects of the mechanism, the inoculated plant will be considered as a non-host because there will only be subliminal infection. Similarly, if the virus is able to suppress the mechanism but cannot block the signal of silencing it is likely that local or systemic spread of the virus will be impaired. Probably the best prospect for understanding this proposed adaptative process involves characterisation of mutants impaired in PTGS (Elmayan et al., 1998) and identification of host components interacting with viral suppressors. In addition, the increasing body of evidence that PTGS

also operates in animals (reviewed in (Hammond et al., 2001)) raises the fascinating possibility that silencing suppression has also been adopted by animal viruses.

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CHAPTER 8

A viral movement protein prevents systemic spread of gene silencing in *Nicotiana benthamiana*

8.1 Abstract

It is shown, here, with potato virus X (PVX) that PTGS operates as a systemic defence system. Our results indicate that in virus-induced PTGS, as shown previously with transgene-induced PTGS, there is a systemic silencing signal that operates in a nucleotide sequence-specific manner and is likely to have a nucleic acid as specificity determinant. However, in grafting experiments or with movement defective forms of PVX we could not detect systemic signaling of PTGS unless the gene for the 25kDa viral movement protein (p25) was modified or removed. Further investigation of p25 suggests that there are two branches in the PTGS pathway that converge in the production of 25 nucleotide RNAs corresponding to the target RNA. One of these branches seems unique to virus-induced PTGS and is not affected by p25. The second branch seems common to both virus- and transgene-induced PTGS and is blocked by p25. This analysis suggests that the signal of systemic PTGS is produced in this second branch of the pathway and is possibly a precursor of the 25 nucleotide RNAs.

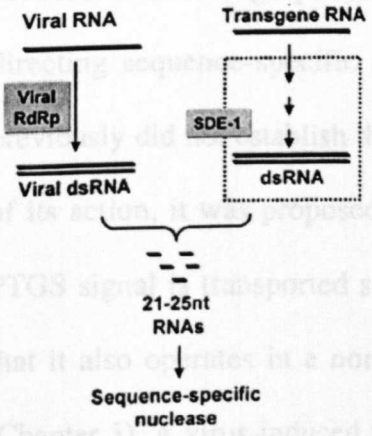
8.2 Introduction

The analyses described in the previous chapters indicate that PTGS in transgenic plants is related to an anti-viral defence system that operates at the level of RNA. PTGS is probably activated when a surveillance system recognises viral or transgene RNA. Subsequent to the recognition event, these RNAs are degraded in a sequence-specific manner. In transformed plants, PTGS is targeted against transcripts of the transgene and any similar endogenous genes so that the corresponding gene products accumulate at a low level. In virus-infected cells, PTGS is targeted against the viral RNA and causes its accumulation to slow down or stop at late stages in the infection process.

The necessity for a specificity determinant in PTGS was discussed in the introduction chapter, and it was proposed that this factor could be constituted of low molecular weight antisense RNA. The work of Andrew Hamilton, in our lab, gave strong support to this hypothesis. He showed that 25 nucleotide (25nt) RNA species corresponding to the target RNA consistently accumulate in plants exhibiting PTGS induced by various sense transgenes or in PVX-infected tissues (Hamilton and Baulcombe, 1999). Similar sized, antisense RNA were also found accumulating to the same abundance as the sense species, suggesting that these RNAs are produced from a dsRNA template (Hamilton and Baulcombe, 1999). Subsequently, 25nt RNA species were detected in animals undergoing RNA interference (RNAi), a PTGS-like mechanism experimentally induced by double-stranded (ds)RNA (Parrish et al., 2000; Zamore et al., 2000). In *Drosophila*, there is strong evidence that 25nt RNA is processed from the dsRNA trigger of RNAi and serves

as guide RNA for a sequence-specific nuclease that executes the degradation step of silencing (Hammond et al., 2000). The fact that sense and antisense small RNA are invariably associated with PTGS, regardless of its initial trigger, also provides support to the previously made suggestion (Chapter 1) that dsRNA, a likely precurors of the 25nt RNA, is an unavoidable step of PTGS.

In plants, the role of PTGS in virus protection is illustrated by the hypersuceptibility of *sde1/sgs2* mutant *Arabidopsis* to cucumber mosaic virus (CMV) (Mourrain et al., 2000). SDE1/SGS2 is a protein similar to RNA-directed-RNA-polymerase and is the first plant component identified to be necessary for sense transgene-mediated PTGS (Dalmay et al., 2000; Mourrain et al., 2000). With caulimo-, nepo-, potex- and tobraviruses, there is evidence that PTGS accounts, at least in part, for cross protection against infection with a second virus (Ratcliff et al., 1999). The first virus induces PTGS so that the infected cells are primed to resist the second virus in a nucleotide-specific manner. Also consistent with the widespread occurrence of PTGS in virus-infected cells, it has been shown in the previous chapters that many viruses are able to overcome or prevent PTGS. Presumably,



Model 8.1. PTGS as a branched pathway in plants. This model is adapted from the work of Dalmay et al., 2000. In the SDE1-independent branch of the PTGS pathway, the viral RNA is copied into double stranded RNA (viral dsRNA) by the virus-encoded RNA-dependent-RNA-polymerase (viral RdRp). Transgene transcripts are processed into dsRNA through a series of steps that involve SDE1. Both viral and transgene dsRNAs are then processed into 21-25nt RNAs, giving specificity to a nuclease that mediates the degradation step of PTGS. In this model, the CMV RNA, as opposed to that of many other viruses, would also enter the SDE1 branch.

silencing is a basic response to virus infection and the production of a suppressor represents a necessary adaptation by the virus if it is to replicate and spread in a plant.

Although *sde1/sgs2* plants are more susceptible to CMV, they are nevertheless as susceptible as the wild type *Arabidopsis* to many other viruses and are not compromised for VIGS of an endogenous phytoene desaturase gene (Dalmay et al., 2000). These resullts led to the suggestion that in plants, there are SDE1-dependent and SDE1-independent branches of the PTGS pathway (Dalmay et al., 2000). Both branches are thought to be dependent on synthesis of dsRNA and to converge at, or before, production of 25nt RNA. The dsRNA in the SDE1-independent branch would be produced through replication of the virus and would thus be dependent on the viral-encoded RdRp (Model 8.1).

One of the most intriguing features of PTGS in transgenic plants is that it is not cell autonomous. It was shown (Chapters 4 and 5) that a signal for gene silencing can move between cells through plasmodesmata and long distances through the vascular system, directing sequence-specific degradation of target RNAs. The experiments described previously did not establish the physical nature of the signal but, based on the specificity of its action, it was proposed that it incorporates a nucleic acid. This discovery that a PTGS signal is transported systemically in transgenic plants prompted the speculation that it also operates in a non-cell autonomous manner during natural virus infections (Chapter 5). A virus-induced silencing signal could migrate cell-to-cell in advance of the infection front and be transported over long distances through the phloem. The effect of

this intercellular signalling would be to potentiate RNA sequence-specific virus resistance in non-infected tissues and, consequently, to delay spread of the virus through the plant.

The experiments described here provide evidence to support this idea, using PVX-based-experimental systems in which movement of a virus-induced signal could be uncoupled from movement of the virus. We show that the antiviral signal molecule can be transported over several centimetres from the infected cells and accumulates in and around the veins of recipient leaves. In the course of developing this study, we discovered that the 25kDa movement protein of PVX (p25) is a suppressor of the systemic PTGS response. Further experiments with p25 allowed us to differentiate two branches of the PTGS pathway. One branch is activated by replicating viral RNA and is not affected by p25. The second branch can be activated by non replicating RNA of viral or transgene origin and is suppressed by p25. Our analysis indicates that the systemic signal of silencing is possibly produced in this second, p25-sensitive branch of the pathway and could be a precursor of the 25 nucleotide RNAs.

8.3 Systemic silencing in PVX-infected tissues

To test the hypothesis that signaling of PTGS is a systemic anti-viral defence we designed grafting experiments in which virus movement would be uncoupled from

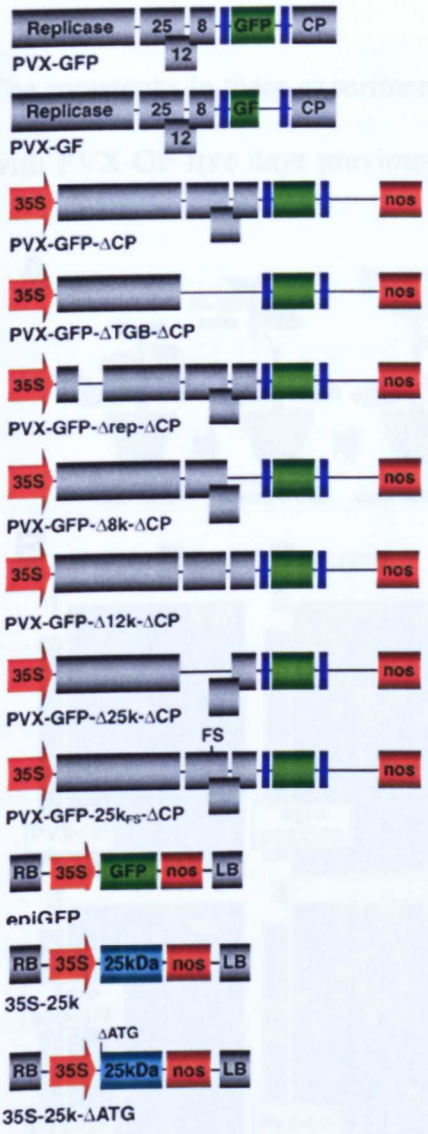


Figure 8.1. Viral and transgene constructs used in this study. PVX-GFP and PVX-GF were described previously in chapter 3, as well as the various open reading frames found in the PVX genome. All other viral constructs were based on the PVX-GFP construct coupled to the 35S promoter and nos terminator and inserted into the T-DNA of the pBin19 binary vector plasmid. PVX-GFP-ΔCP carries a deletion spanning the entire CP ORF; PVX-GFP-ΔTGB-ΔCP and PVX-GFP-Δrep-ΔCP are based on PVX-GFP-ΔCP and carry a deletion spanning all the TGB ORFs and an in-frame deletion in the replicase ORF, respectively. The PVX-GFP-Δ12k-ΔCP and PVX-GFP-Δ25k-ΔCP constructs carry deletions into the 12kDa and 25kDa ORF, respectively. PVX-GFP-Δ8k-ΔCP carries a frameshift mutation that prevents translation of the 8kDa ORF. PVX-GFP-$25k_{FS}$-ΔCP carries a frameshift mutation in the 25kDa ORF, indicated by "FS" (see "experimental procedures" for details). The epiGFP construct was described previously. The 35S-25k and 35S-25k-ΔATG constructs are based on the PVX 25kDa ORF coupled to the 35S promoter and the 35S terminator and inserted into the T-DNA of pBin19. The start codon of the 25kDa ORF has been removed in 35S-25k-ΔATG, as indicated by "ΔATG". LB and RB indicate left and right border of the pBin19 T-DNA, respectively.

transport of a silencing signal. The experiments used line 16c of *Nicotiana benthamiana* carrying a highly expressed green fluorescent protein (GFP) transgene (Chapter 3). Transgene-induced, systemic silencing of the GFP transgene was initiated as described

(Chapters 4 and 5) by localized infiltration of the epiGFP strain of *Agrobacterium* carrying the epiGFP construct (Chapter 5, section 5.3.1). Virus-induced PTGS of the GFP transgene was initiated by infection with the PVX-GF vector described in Chapter 3 (See also Figure 8.1).

The rootstocks in these experiments were GFP transgenic plants that had been inoculated with PVX-GF five days previously. These plants exhibited the early signs of PTGS of

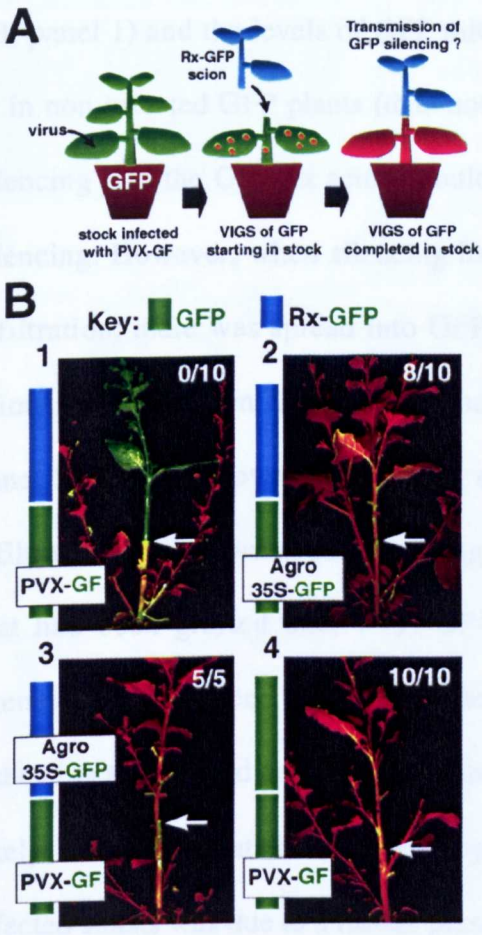


Figure 8.2. Test for graft-transmission of PVX-GFP-induced signal in GFP transgenic *N. benthamiana*. (**A**) Schematic description of the grafting experiments. (**B**) Young GFP transgenic rootstocks were either inoculated with PVX-GF (panels 1,3,4) or infiltrated with the epiGFP strain of *Agrobacterium* (panel 2). Five days later, transgenic scions carrying either the GFP transgene (panel 4,) or both the GFP and the *Rx* transgenes (*Rx*-GFP, panels 1, 2, 3) were wedge-grafted onto the rootstocks. Graft-transmission of GFP silencing was then scored under UV illumination throughout time (scores are depicted in the right-hand top corner of each panel). The photographs in panels 1-4 were taken 4 weeks after grafting. The arrow indicates the graft union. Note that GFP can appear yellow because of the long exposure times required for imaging.

GFP (Figure 2A). The scions carried a GFP transgene together with the *Rx* gene that confers extreme resistance against PVX (Bendahmane et al., 1999). The presence of *Rx* would prevent replication of PVX-GF in the scions but should have no effect on systemic

transport of a silencing signal (Figure 2A). By 20 days after grafting, PTGS of GFP was extensive in the rootstocks, as indicated by loss of green fluorescence under UV illumination (Figure 2B, panel 1). As expected, there was no spread of PVX-GF into the *Rx*/GFP scions, indicated by the absence of PVX symptoms and the failure to detect PVX-GF RNAs by Northern analysis (data not shown). Also as expected, there was spread of PVX-GF and of gene silencing into the GFP scions without *Rx* (Figure 2B, panel 4, data not shown). However, there was no systemic silencing of GFP in the *Rx*/GFP scions in any of ten grafts tested. The scions remained green fluorescent (Figure 1B, panel 1) and the levels of GFP mRNA, as assessed by Northern analysis, were high as in non-infected GFP plants (data not shown). The absence of systemic spread of GFP silencing into the GFP/*Rx* scions could result if *Rx* was able to interfere with systemic silencing. However, when silencing had been induced in the stocks by *Agrobacterium* infiltration, there was spread into GFP/*Rx* scions in 8 out of 10 graftings tested: these scions had lost green fluorescence and GFP mRNA could not be detected (Figure 2B, panel 2, data not shown). In a further control to assess the effect of *Rx* on silencing we infiltrated *Agrobacterium* cells carrying the epiGFP construct directly into GFP/*Rx* scions that had been grafted onto PVX-GF-infected plants. In all five of these tests GFP silencing was induced and spread through the GFP/*Rx* scions (Figure 2B, panel 3) indicating that *Rx* had no effect either on initiation or systemic spread of PTGS. It seemed likely, therefore, that the failure of systemic silencing to spread out of the PVX-GF-infected stocks was due to a factor, presumably a protein, encoded in the PVX genome.

8.4. PVX interferes with systemic silencing

To assess the possibility that PVX is able to block movement of a silencing signal, we infiltrated *Agrobacterium* strain epiGFP (Figure 8.1) into leaves of GFP plants that had been previously infected with wild type PVX. We reasoned that a PVX-mediated block on signal movement would prevent or interfere with the spread of the *Agrobacterium*-induced silencing out of the infiltrated leaves. The controls in these experiments were

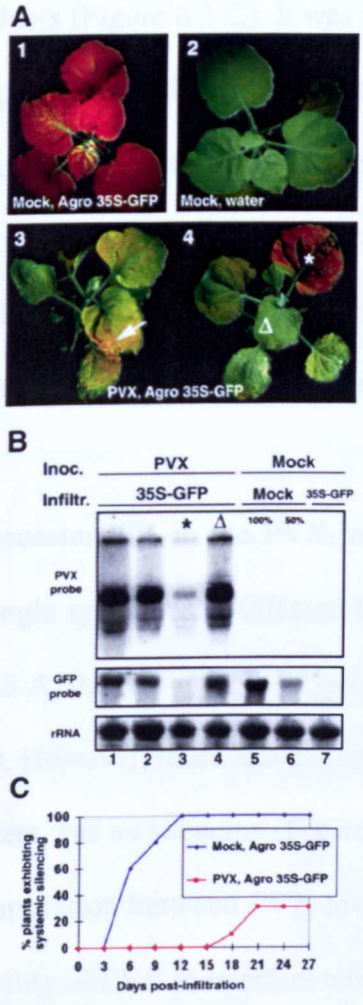


Figure 8.3. PVX interferes with systemic movement of a GFP silencing signal. (**A**) Spreading of the silencing signal in PVX-infected GFP plants. Non-silenced GFP seedlings were first inoculated with either water (panels 1 and 2) or wild type PVX (panels 3 and 4). After systemic infection, an upper leaf was infiltrated with the 35S-GFP strain of agrobacterium (panels 1,3,4) or with water (panel 2). Systemic silencing was then assessed under UV illumination. The photographs were taken at 21 days post-infiltration and represent typical plants for each treatment. The arrow in panel 3 shows restriction of systemic silencing in the veins of a single leaf. The * and Δ symbols in panel 4 indicate leaf samples that were used for the Northern analysis depicted in (B). (**B**) Northern analysis of PVX and GFP mRNAs. Total RNA was extracted at 21 days post infiltration from systemic leaves of GFP plants that had received one of the treatments described in (A). Northern analysis of 10 μg of RNA was carried out to detect accumulation of the PVX and GFP RNA, using a probe corresponding to the full length PVX and the full-length GFP cDNA, respectively. Lanes 1 and 2 show hybridisation of RNA from tissues of independent plants; lanes 3 and 4 show hybridisation of RNA extracted from two leaves (marked * and Δ) of the plant pictured in panel A4. Lane 6 shows a one-half dilution of the RNA assayed on lane 5 into total RNA extracted from a nontransformed plant. Ethidium bromide staining of rRNA in the electrophoresed gel shows relative RNA loadings. (**C**) Kinetics of GFP systemic silencing. Each point in the graph represents the percentage of plants exhibiting GFP systemic silencing as assessed under UV illumination. The average values are from 30 individual plants tested in 3 independent experiments, for each treatment. Plants were scored as silenced even if the systemic silencing was confined to small areas near the veins of a few leaves (i.e. panel A3). Systemic silencing in mock-inoculated plants was always extensive (i.e. panel A1).

mock-, as opposed to PVX- inoculated GFP plants. Systemic silencing in these control plants was initiated at 5-6 days post infiltration and by 21 days, there was extensive loss of GFP fluorescence (Figures 8.3-A panel 1; Figure 8.3-C). Accordingly, the GFP mRNA was below the detection level in 100% of the plants tested (Figure 8.3-B, lane 7, GFP probe). In contrast, systemic silencing was initiated in only 30% of the PVX-infected plants (Figure 8.3-C). It was striking that the GFP silencing in these PVX-infected plants spread more slowly than in mock-inoculated controls and was restricted to small areas around the veins of some upper leaves (Figure 8.3-A, panel 3, arrow). From these results we conclude that PVX was able to interfere with systemic silencing . Confirming this idea we found that the GFP mRNA levels in the PVX-infected leaves were almost as high as in non silenced control plants (Figure 8.3-B, lanes 1, 2 and 4 compared to lane 5).

Occasionally, in the PVX-infected plants, there was extensive silencing of GFP on a single systemically infected leaf. These leaves had low levels of GFP fluorescence (Fig 8.3-A, the leaf marked "*" in panel 4) and low levels of GFP mRNA (Figure 8.3-B, lane 3). However, these leaves contained a lower level of PVX RNA than the leaves in which there was no silencing (Figure 8.3-B, lane 3 compared with lanes 1,2 and 4). This inverse correlation between PVX levels and PTGS was therefore consistent with the proposed ability of PVX to interfere with systemic silencing.

8.5. The PVX-encoded 25kDa protein prevents systemic silencing

To determine whether PVX-encoded proteins are able to prevent or interfere with systemic silencing we carried out experiments with deletion mutants of PVX-GFP

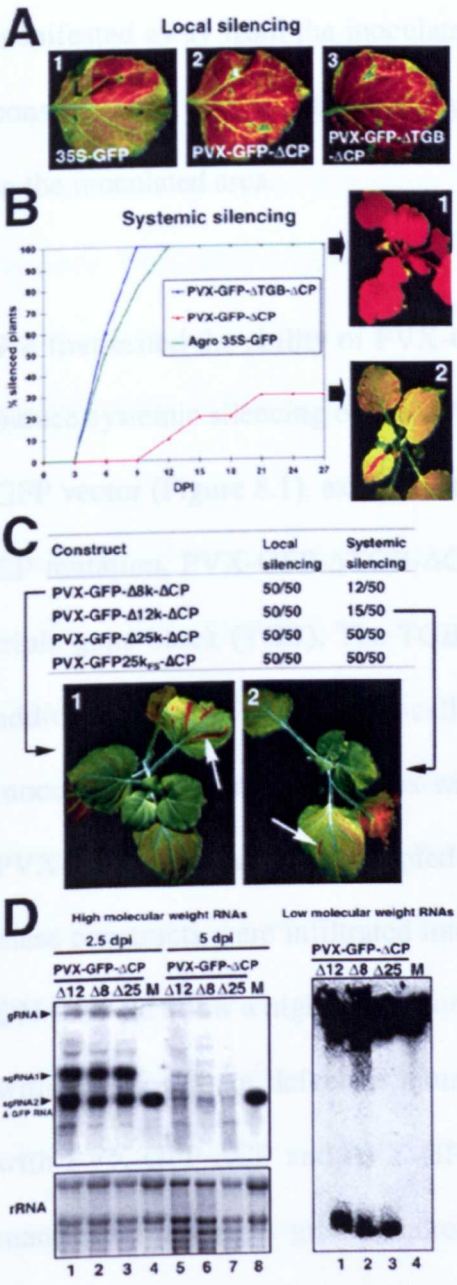


Figure 8.4. Effect of the TGB proteins on systemic movement of the GFP silencing signal. (**A**) Following strong transient expression of GFP, the area infiltrated with either 35S-GFP, PVX-GFP-ΔCP and PVX-GFP-ΔTGB-ΔCP become progressively red-fluorescent under UV illumination. The photos in panels 1-3 were taken at 12 days post-infiltration and suggest that GFP silencing was initiated to the same extent by either constructs. (**B**) Kinetics of GFP systemic silencing. Each point in the graph represents the percentage of plants exhibiting GFP systemic silencing as assessed under UV illumination. The average values are from 30 individual plants tested in 3 independent experiments, for each treatment. Plants were scored as silenced even if the systemic silencing was confined to small areas near the veins of a few leaves (i.e. panel B2, at 21 dpi). All of the PVX-GFP-ΔTGB-ΔCP-inoculated plants showed extensive systemic silencing at 21dpi (i.e. panel B1). Note that GFP can appear yellow because of the long exposure times required for imaging. (**C**) GFP systemic silencing induced by individual TGB mutants of PVX-GFP-ΔCP. The number of plants exhibiting local and systemic silencing of GFP is indicated alongside the total number of plants tested in 3 independent experiments. The arrows show restriction of systemic silencing in the veins of single leaves of PVX-GFP-Δ8k-ΔCP and PVX-GFP-Δ12k-ΔCP inoculated plants, at 21 dpi. Plants exhibiting such phenotype were scored as silenced in the assay. Systemic silencing induced by PVX-GFP-25k$_{FS}$-ΔCP and PVX-GFP-Δ25k-ΔCP was always extensive (i.e. panel B1). (**D**) Northern analysis of high and low molecular weight RNAs. Total RNA was extracted at 2.5 and 5dpi from leaves of GFP plants that had been infiltrated with either PVX-GFP-Δ8k-ΔCP, PVX-GFP-Δ12k-ΔCP, PVX-GFP-Δ25k-ΔCP, or water (Mock: M). Northern analysis was carried out on 10μg of the high molecular weight RNA fraction, to detect accumulation of the PVX-GFP and transgene GFP RNA, using a probe corresponding to the central region of the GFP cDNA (Left panel). Ethidium bromide staining of the electrophoresed gel shows rRNA loading. Northern analysis of the low molecular weight fraction (right panel) was carried out to detect accumulation of 22-25nt antisens GFP RNAs. Loading in lanes 1-4 was standardized with ethidium bromide staining and quantification of tRNAs in each sample. The probe used corresponded to the full-length GFP cDNA.

(Figure 8.1). These mutant viruses would have been confined to the initially infected cell because they were all defective for the coat protein (CP) that is required for cell-to-cell and long distance movement of PVX. If, as predicted, a PVX-encoded protein prevented systemic silencing, PTGS initiated by the corresponding PVX-GFP mutant would be manifested away from the inoculated cells. In contrast, silencing initiated by PVX-GFP constructs carrying mutations in any other open reading frame (ORF) would be restricted to the inoculated area.

We first tested the ability of PVX-GFP-ΔCP and PVX-GFP-ΔTGB-ΔCP (Figure 8.1) to induce systemic silencing of the GFP transgene. These constructs are similar to the PVX-GFP vector (Figure 8.1), except that there is a deletion in the CP ORFs. In addition to the CP mutation, PVX-GFP-ΔTGB-ΔCP carries a deletion spanning all three ORFs of the triple gene block (TGB). The TGB encodes three proteins that are strictly required, in addition to the CP, for cell-to-cell movement of PVX. In order to generate high titer inocula of these mutant viruses we used the pBin19 Ti-plasmid vectors in which the PVX-GFP constructs were coupled to a 35S promoter. *Agrobacterium* cultures carrying these constructs were infiltrated into leaves of GFP transgenic plants. Transfer of the T-DNA would allow a high proportion of cells inside the infiltrated area to become infected with the movement defective mutants of PVX-GFP. At 3 days post inoculation (dpi), with PVX-GFP-ΔCP and PVX-GFP-ΔTGB-ΔCP, there was strong expression of GFP manifested as bright green fluorescence in the infiltrated regions (data not shown). However, starting at 5-6dpi, the infiltrated regions became red-fluorescent, suggesting that local PTGS of GFP had been initiated by both of these constructs (Figure 8.4-A,

panels 2-3). The development of this local silencing was as rapid as in leaves infiltrated with the 35S-GFP construct (Figure 8.4-A, panel1). With both PVX-GFP-ΔTGB-ΔCP and epiGFP, systemic silencing was initiated in 100% of the GFP plants and developed as quickly and extensively as with the epiGFP construct (Figure 8.4-B, graph and panel 1). In contrast, systemic silencing initiated with the PVX-GFP-ΔCP construct was delayed (Figure 8.4-B, graph), appeared in only 30% of the inoculated plants and, in those plants, was restricted to the veins in a few leaves (Figure 8.4-B, panel 2). Because the difference between PVX-GFP-ΔTGB-ΔCP and PVX-GFP-ΔCP involved the TGB ORFs, these results suggested that one or more of the TGB proteins prevented systemic silencing from the PVX-GFP-ΔCP-infected cells.

Similar experiments were carried out with PVX-GFP-ΔCP derivatives in which the TGB ORFs were mutated individually (PVX-GFP-Δ25k-ΔCP, PVX-GFP-$25k_{FS}$-ΔCP, PVX-GFP-Δ12K-ΔCP and PVX-GFP-Δ8K-ΔCP; Figure 8.1). With all of these mutants, the infiltrated region became red-fluorescent, suggesting that there was initiation of local PTGS of GFP (Figure 8.4-C). However, the only TGB mutants that produced extensive systemic silencing were those carrying either a deletion (PVX-GFP-Δ25k-ΔCP, Figure 1) or a frameshift mutation (PVX-GFP-$25k_{FS}$-ΔCP, Figure 8.1) in the ORF of the 25kDa protein (p25) (Figure 8.4-C). The viruses carrying mutations in the ORFs of the 12kDa and 8kDa proteins (PVX-GFP-Δ12K-ΔCP and PVX-GFP-Δ8K-ΔCP, respectively, Figure 1) encode a functional 25kDa protein and, like PVX-GFP-ΔCP were poor inducers of systemic silencing. Most of the GFP plants inoculated with these constructs did not exhibit any systemic silencing of GFP (Figure 8.4-C). However, as with PVX-GFP-ΔCP,

about 25% of the inoculated plants exhibited partial silencing of GFP. At 21 dpi this partial silencing was restricted to the regions in and around the veins of some upper leaves (Figure 8.4-C, arrows in panels 1 and 2) and did not develop further.

In principle, the contrasting silencing phenotypes triggered by the PVX-GFP TGB mutants could be a direct effect of p25. Alternatively, there could be an indirect effect if the mutations affected replication or the ability of these mutants to induce PTGS of GFP in the inoculated leaves. To resolve these alternatives we carried out northern analysis of RNA from the infiltrated leaf tissues at 2.5 and 5dpi, using a GFP-specific probe. At 2.5 dpi, with PVX-GFP-Δ12K-ΔCP, PVX-GFP-Δ8K-ΔCP and PVX-GFP-Δ25k-ΔCP, the extracts contained four major RNA species (Figure 8.4-D, left panel, tracks 1-3) detected with the GFP probe. The genomic viral RNA (gRNA) was the least and the viral subgenomic (sg) RNA1 was the most abundant. The sgRNA2 co-migrated with and could not be differentiated from the GFP transgene mRNA (Figure 8.4-D, left panel, tracks 1-4). At 2.5 dpi, these RNAs were all abundant in the PVX-GFP-Δ12K-ΔCP, PVX-GFP-Δ8K-ΔCP and PVX-GFP-Δ25k-ΔCP-infected tissues. At 5dpi, however, with all three TGB mutants, the levels of these RNA species were markedly reduced. This reduction was dependent on the virus because, in mock inoculated tissue, the GFP mRNA was at the same level at 2.5 and 5dpi (Figure 8.4-D, left panel, tracks 4 and 8). Thus, this change in RNA abundance was likely due to PTGS that was targeted against both viral and transgene GFP RNA species (Figure 8.4-D, left panel, tracks 5-8, sgRNA3 & GFP RNA).

As an additional test of PTGS induced by the TGB mutants we assayed for 22-25nt antisense GFP RNAs at 5dpi. In other systems, the relative amount of those small antisense RNAs correlates with the level of PTGS. As expected, these 22-25nt GFP RNAs were absent in the extract of mock-infiltrated leaves (Figure 8.4-D, right panel, track 4). However, in PVX-GFP infected tissues these RNAs were present and their levels were unaffected by mutations in the TGB ORFs (Figure 8.4-D, right panel, tracks 1-3). This data indicate that all three TGB mutants were efficient inducers of PTGS of GFP. Combined, these results show that all of the TGB mutants replicated and activated intracellular PTGS to a similar extent. However, systemic spread of silencing only occurred when the PVX-GFP constructs carried mutations in the p25 ORF. It is unlikely that this block was an RNA-mediated effect because systemic silencing was initiated by a PVX-GFP mutant with a frame-shift mutation in the p25 ORF. Therefore, we conclude that the p25 protein was able to prevent systemic PTGS of the GFP transgene.

8.6. PVX replication enhances production of the GFP systemic silencing signal

If systemic silencing is a manifestation of an antiviral defense system, the PVX-GFP-ΔTGB-ΔCP construct should produce more silencing signal than similar constructs lacking the PVX replication function. However, we could not test this prediction using *Agrobacterium* inoculation of viral constructs because systemic silencing was induced with high efficiency by many GFP constructs, irrespective of whether they encoded replicating RNAs, as shown previously (Chapter 5).

The constructs described so far (Figures 8.1) were all assembled in a pBin19 vector plasmid. However, we showed in that Agrobacterium strains carrying the epiGFP construct in a pSLJ background rather than pBin19 were not able to induce systemic silencing (Chapter 5). As explained, the pSLJ plasmids accumulate in *Agrobacterium* at lower copy number than the pBin19 vectors, and it is likely that this feature reduces the efficiency of T-DNA transfer from the bacterium to the plant cell. Effective T-DNA transfer is a prerequisite for activation of systemic silencing; for example, omission of acetosyringone in the bacterium suspension) leads to a dramatic reduction of systemic silencing activation (Chapter 5). Thus, it seemed likely that a pSLJ-based system could be used to test the importance of PVX-GFP replication in triggering systemic silencing of GFP.

Construct in pSLJ	GFP expression in NT plants	Systemic silencing at 9 DPI	Systemic silencing at 27 DPI
35S-GFP	(+)	0/30	0/30
PVX-GFP-ΔCP	+	0/30	5/30 (veins only)
PVX-GFP-ΔTGB-ΔCP	+	27/30 (veins)	30/30 (complete)
PVX-GFP-Δrep-ΔTGB-ΔCP	-	0/30	0/30

Table 8.1. Induction of systemic silencing by PVX-GFP derivatives produced from the pSLJ binary vector T-DNA. The values presented were collected in 3 independent experiments involving 10 plants each. DPI: days-post-infiltration; (+): faint GFP fluorescence observed in transient expression; +: strong GFP fluorescence observed in transient expression.

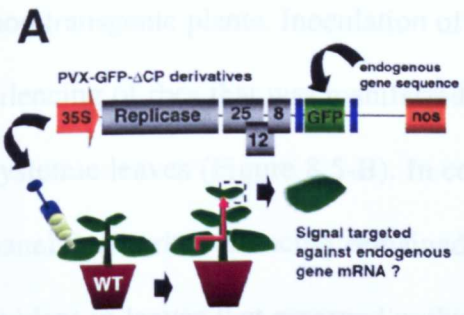
To carry out these tests we transferred the PVX-GFP-ΔCP and PVX-GFP-ΔTGB-ΔCP constructs (Figure 8.1) into pSLJ. The cloning of the epiGFP construct into pSLJ was described in Chapter 5. In addition, PVX-GFP-Δrep-ΔTGB-ΔCP carrying an in frame inactivating mutation in the replicase ORF was constructed. These constructs were

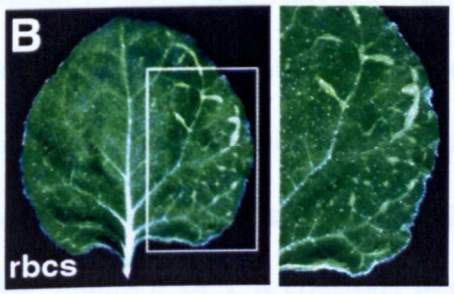
inoculated by *Agrobacterium* infiltration, as in the experiments described in Figures 8.2-8.4. Initially, at 2 days post-infiltration, the pSLJ versions of PVX-GFP-ΔCP and PVX-GFP-ΔTGB-ΔCP induced bright green fluorescence in inoculated cells, whereas the pSLJ version of epiGFP caused faint green fluorescence. This production of GFP from the viral constructs was dependent on PVX replication because there was no green fluorescence when the *Agrobacterium* strain carried PVX-GFP-Δrep-ΔTGB-ΔCP (Table 8.1). By 9 days post-infiltration, in the PVX-GFP-ΔTGB-ΔCP inoculated plants, there was systemic silencing that eventually spread throughout the plant. In contrast, there was no extensive systemic silencing triggered by PVX-GFP-ΔCP, PVX-Δrep-GFP-ΔTGB-ΔCP, or epiGFP. With PVX-GFP-ΔCP this was likely because the 25kDa TGB protein had blocked spread of the signal, as described above. However, as PVX-GFP-Δrep-ΔTGB-ΔCP does not encode the 25kDa protein the failure to induce systemic silencing must be due to the absence of PVX RNA replication.

8.7. Systemic silencing in non transgenic plants

The experiments described above were not directly informative about the extent of systemic silencing in virus-infected plants because they involved GFP transgenes integrated in the plant genome and in the T-DNA of the infiltrated *Agrobacterium*. Any virus-induced effects would have been amplified and relayed by these transgenes, as shown previously (Chapter 5), so that systemic silencing would have been more extensive than in non transgenic plants. Therefore, to obtain a more accurate picture of the systemic signaling due to virus infection a series of experiments was carried out in non transgenic plants. The PTGS in these experiments was targeted against the endogenous gene

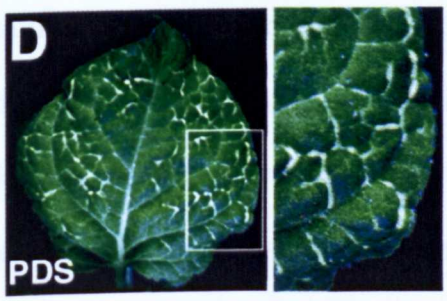
encoding the ribulose bisphosphate carboxylase small subunit (rbcs). As shown previously (Jones et al., 1999), this gene is a potential target of PVX-induced PTGS but, unlike transgenes, it does not participate in the initiation, amplification or maintenance of the mechanism. Therefore, it was likely that systemic silencing of rbcs would indicate the extent of signal spread from the virus-infected cells (Figure 8.5-A). The constructs in these experiments were derivatives of PVX-GFP-ΔCP (Figure 8.1) in which a 500nt fragment of the rbcs cDNA was inserted into the GFP ORF (Figure 8.5-A). These derivatives are collectively referred to as PVX-rbcs-X in which "X" indicates the various mutations carried by each individual construct (Figure 8.5-C).





C

Construct	rbcs systemic silencing
PVX-rbcs-ΔTGB-ΔCP	35/40
PVX-rbcs-Δrep-ΔTGB-ΔCP	0/40
PVX-rbcs-Δ12k-ΔCP	0/40
PVX-rbcs-Δ25k-ΔCP	36/40



E

Construct	PDS systemic silencing
PVX-PDS-ΔTGB-ΔCP	35/40
PVX-PDS-Δrep-ΔTGB-ΔCP	0/40

Figure 8.5. Systemic silencing in non transformed plants. (**A**) The diagram summarizes the order of events described in (B) and (D). (**B**) Systemic silencing of rbcs. First, one or two expanded leaves of a non transformed seedling were infiltrated with a strain of *Agrobacterium* containing either PVX-GFP-ΔTGB-ΔCP or PVX-GFP-Δrep-ΔCP (Figure 1) in which a 500-nucleotide fragment from the ribulose bisphosphate carboxylase small subunit (rbcs) cDNA was inserted in the GFP ORF. Fourteen days later, systemic, new emerging leaves were inspected for silencing of rbcs. The picture represents a typical systemic leaf from a plant inoculated with the PVX-rbcs-ΔTGB-ΔCP derivative showing yellow-green chlorosis in and near the class II and III veins. (**C**) Influence of p25 and PVX replication on systemic silencing of rbcs. The table indicates the number of plants exhibiting systemic silencing of rbcs and the total number of plants tested for each construct. (**D**) Systemic silencing of PDS. The principle of the experiment is similar to that in (A-B) except that a 415-nucleotide fragment from the central region of the phytoene desaturase (PDS) cDNA was inserted into the GFP ORF of the corresponding PVX-GFP derivative (Figure 1). The picture represents a typical systemic leaf from a plant inoculated with the PVX-PDS-ΔTGB-ΔCP derivative and shows photobleaching associated with PDS silencing near the class II and III veins. (**E**) Influence of PVX replication on systemic silencing of PDS. The table indicates the number of plants exhibiting systemic silencing of PDS and the total number of plants tested for each construct.

The *Agrobacterium* infiltration procedure was used to inoculate these PVX constructs to non transgenic plants. Inoculation of the PVX-rbcs-ΔTGB-ΔCP derivative led to systemic silencing of rbcs that was manifested as yellow-green chlorosis in and around the veins of systemic leaves (Figure 8.5-B). In contrast to the extensive GFP silencing (Figure 8.4-B, panel1), the rbcs silencing remained restricted to the vicinity of the veins and was only evident in leaves that emerged within 10-16 dpi. This phenotype was consistent with the lack of relay-amplification associated with PTGS of the rbcs gene and was likely a direct indicator of the virus-induced signal. As with GFP silencing, the rbcs systemic effect required mutation of the 25kDa ORF (in PVX-rbcs-ΔTGB-ΔCP and PVX-rbcs-Δ25k-ΔCP, Figure 8.5-C). A construct in which the 25kDa ORF was intact (PVX-rbcs-Δ12K-ΔCP) did not induce systemic silencing (Figure 8.5-C). From these results we conclude that, in the absence of a transgene, a virus-induced silencing signal can move several centimeters from infected cells and is primarily localized in the vicinity of the veins. Importantly, the replication-defective PVX-rbcs-Δrep-ΔCP failed to induce systemic silencing of rbcs (Figure 8.5-C). This result suggests that in non transgenic plants, production of the signal is dependent on the replication competence of the viral genome responsible for its induction.

Similar results were obtained with PVX-GFP-ΔCP derivatives targeted against the phytoene desaturase (PDS) gene. As for rbcs, the systemic silencing of PDS, manifested as photobleaching, was only transient and localized around the veins of some new emerging leaves (Figure 8.5-D). It was also dependent on PVX replication (Figure 8.5-E). The PDS mRNA is several orders of magnitude less abundant than the rbcs mRNA (data

no shown). We can therefore rule out that the level of target gene expression influenced the vein pattern and persistence of systemic silencing in non transgenic plants.

The insertion of the PDS and rbcs fragments into the GFP ORF carried by the PVX derivatives depicted in Figure 8.5 was further exploited to assess the antiviral function of systemic signalling. It was predicted that the systemic silencing signal activated by PVX would not only be targeted against the endogenous gene sequences but also against the surrounding GFP sequences. As GFP is absent in the upper silenced tissues and as it is only represented in the viral genome, silencing of GFP in systemic tissue would be a direct indicator that the signal possess an antiviral function. The systemic silencing of GFP in these NT plants was diagnosed with a second *Agrobacterium* infiltration into the leaves exhibiting systemic PDS silencing. This second infiltration was with a strain carrying the epiGFP construct (Figure 8.6-A). In control plants that had been inoculated with PVX-PDS-Δrep-ΔTGB-ΔCP, the expression of the GFP reporter was uniformly distributed in the infiltrated zone of the leaf (Figure 8.6-B panels 1-4). In contrast, in plants that had been inoculated with PVX-PDS-ΔTGB-ΔCP, there was little or no green fluorescence in the regions in and around the veins of leaves exhibiting systemic silencing of PDS (Figure 8.6-C panels 2-4). We could rule out that the absence of GFP fluorescence was a secondary effect of PDS silencing because the absence of green fluorescence and the photobleached regions did not always coincide. As shown in Figure 8.6-C (panels 5 and 6) there were often photobleached zones that were fully green fluorescent and, conversely, unbleached regions in which GFP was silenced. Combined, these data indicate that replication of PVX induced a sequence-specific silencing signal

that could spread and trigger PTGS at sites up to 15 centimetres from the site of inoculation.

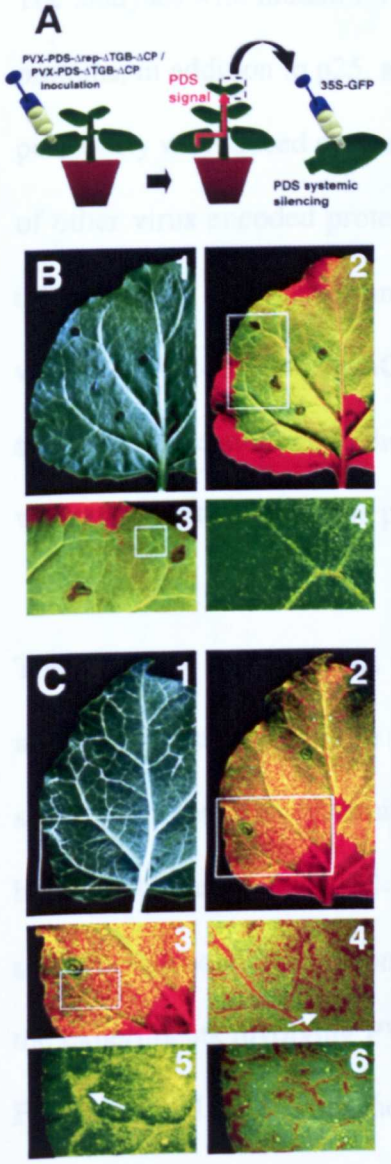
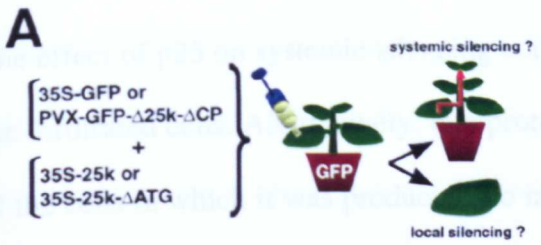


Figure 8.6. PVX-GFP replication induces systemic silencing of GFP in NT plants. (**A**) The diagram summarises the order of events described below. First, systemic silencing of PDS was induced as described in Figure 8.5-B. A developing leaf exhibiting vein photobleaching was then infiltrated with a strain of *Agrobacterium* containing the 35S-GFP construct (Figure 1A). Transient expression of GFP was subsequently monitored under UV illumination, at 3 days post-infiltration. Similar leaves from plants inoculated with PVX-PDS-Δrep-ΔTGB-ΔCP were used as control. (**B**) Transient expression of GFP in leaves of PVX-PDS-Δrep-ΔTGB-ΔCP inoculated plants. Panel 1 is a photograph of the abaxial side of the infiltrated leaf, taken under normal light. Panels 2-4 are views of the same leaf under UV illumination and show uniform transient expression of GFP. (**C**) Transient expression of GFP in leaves of PVX-PDS-ΔTGB-ΔCP inoculated plants. Panel 1 is a photograph of the abaxial side of the infiltrated leaf, taken under normal light. The vein network appears in white because of PDS silencing. Panels 2-4 are views of the same leaf under UV illumination and show exclusion of GFP expression in and around the veins. Silencing of GFP is particularly pronounced in the blind endings of the class III veins (panel 4, arrow). Panels 5 and 6 show that PDS silencing (panel 5, the arrow indicate an island of photobleached tissue observed under normal light) and GFP silencing (panel 6, UV illumination of panel 5) do not always coincide.

8.8. Ectopic expression of p25 and systemic silencing

The analyses with mutant PVX (Figures 3 and 4) did not rule out that other virus-encoded proteins, in addition to p25, are involved in preventing systemic silencing. To address this possibility we induced systemic silencing in the presence of p25 expressed independently of other virus encoded proteins (Figure 8.7-A). Induction of systemic silencing was by infiltration of Agrobacterium strains carrying the epiGFP construct or, as a reference, with the PVX-GFP-Δ25k-ΔCP construct (Figure 8.1). These strains were mixed with a second strain containing either the 35S-25k construct or the 35S-25k-ΔATG construct in which the start codon of the p25 ORF is removed (Figure 8.1).

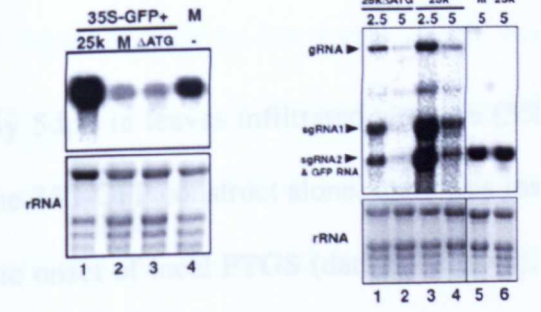
The construct combinations with 35S-25k-ΔATG induced systemic silencing as rapidly and as extensively as with the 35S-GFP construct alone (Figure 8.7-B and data not shown). In contrast, systemic silencing of GFP occurred in only a few plants that had been infiltrated with the 35S-25k combinations (Figure 8.7-B). Moreover, in those plants, systemic silencing was incomplete and was restricted to the veins of a few leaves, as in the experiments involving PVX-GFP derivatives with an intact p25 ORF (Figure 8.7-B, Figure 3B and 3C). From these results we conclude that, of the PVX-encoded proteins, p25 was sufficient to interfere with systemic silencing of the GFP transgene.



B

35S-GFP	
co-infiltrated construct	systemic silencing
35S-25k	4/40 (veins)
35S-25k-ΔATG	37/40 (full)

PVX-GFP-Δ25k-ΔCP	
co-infiltrated construct	systemic silencing
35S-25k	1/20 (veins
35S-25k-ΔATG	20/20 (full)



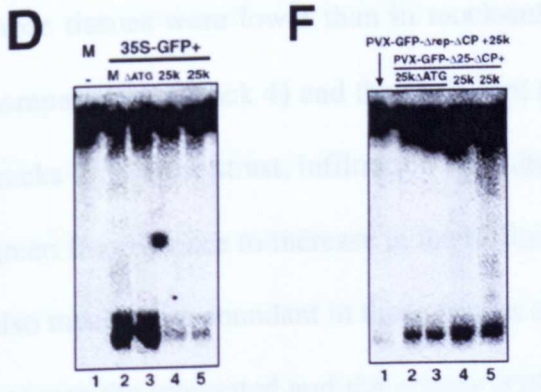


Figure 8.7 Ectopic expression of p25. (**A**) Principle of the experiments described below. A culture of an *Agrobacterium* strain containing the 35S-25k or the 35S-25k-ΔATG construct (Figure 8.1) was mixed (equal volume) with a culture of an *Agrobacterium* strain containing either the 35S-GFP or the PVX-GFP-Δ25k-ΔCP construct (Figure 8.1). The corresponding suspension was then infiltrated into one or two leaves of a young GFP transgenic seedling, and the onset of local and systemic silencing of the GFP transgene was monitored throughout time. (**B**) Systemic silencing of GFP induced with the 35S-GFP transgene (left table) or with PVX-GFP-Δ25k-ΔCP (right table) in combination with 35S-25k or 35S-25k-ΔATG. The values are from independent experiments involving 10 plants each. "Veins" indicates that systemic silencing was only manifested in the veins of a few leaves at 21dpi. "Full" indicates extensive systemic silencing of GFP at 21 dpi. (**C**) Northern analysis of high molecular weight RNAs. Total RNA was extracted at 2.5 and 5dpi from leaves of GFP plants that had been infiltrated with the 35S-GFP construct in combination with either the 35S-25k construct (25k), the 35S-25k-ΔATG construct (ΔATG), or water (Mock: M). Northern analysis was carried out on 10μg of the high molecular weight RNA fraction, to detect accumulation of the GFP RNA, using a probe corresponding to the full length GFP cDNA. Ethidium bromide staining of the electrophoresed gel shows rRNA loading. (**D**) Northern analysis of low molecular weight RNAs was carried out to detect accumulation of 22-25nt antisens GFP RNAs in the 5dpi samples analyzed in (C). Loading in lanes 1-5 was standardized with ethidium bromide staining and quantification of tRNAs in each sample. The probe used corresponded to the full-length GFP cDNA. (**E**) Northern analysis of high molecular weight RNAs. Total RNA was extracted at 2.5 and 5dpi from leaves of GFP plants that had been infiltrated with the PVX-GFP-Δ25k-ΔCP construct in combination with either the 35S-25k construct (25k), the 35S-25k-ΔATG construct (ΔATG), or water (Mock: M). Northern analysis was as described in (C).(**F**) Northern analysis of low molecular weight RNAs. This analysis was performed at 5 dpi, as described in (D). Track 1 shows low levels of 25nt RNAs accumulating in tissues from leaves inoculated with the non replicating PVX-GFP-Δrep-ΔCP in combination with 35-25k.

8.9. Ectopic expression of p25 and local silencing

The effect of p25 on systemic silencing could result from a block of signal production in the infiltrated cells. Alternatively, this protein could prevent movement of the signal out of the cells in which it was produced. To investigate these alternatives we monitored the local effects of p25 on RNA levels and GFP fluorescence in the leaves where PTGS had been initiated. If signal movement was targeted, the local silencing in inoculated cells would be unaffected. However, an effect of p25 on signal production would likely affect initiation of local silencing.

By 5dpi, in leaves infiltrated with the (35S-25k-ΔATG+35S-GFP) combination or with the 35S-GFP construct alone, there was loss of GFP fluorescence, as expected, indicating the onset of local PTGS (data not shown). Correspondingly, the levels of GFP RNAs in those tissues were lower than in mock-infiltrated tissues (Figure 8.7-C, tracks 2 and 3 compared with track 4) and the GFP 25nt antisense RNAs were abundant (Figure 8.7-D, tracks 2-3). In contrast, infiltration with the (35S-25k+35S-GFP) combination caused the green fluorescence to increase in the infiltrated leaf (data not shown). The GFP RNA was also much more abundant in those tissues than in the mock-infiltrated tissues, presumably because the integrated and the ectopic 35S-GFP transgenes were both expressed (Figure 8.7-D, track 1 compared to track 4). Correspondingly, the GFP 25nt antisense RNAs were more than five times less abundant than in tissues infiltrated with 35S-GFP or with (35S-GFP+35S-25k-ΔATG) (Figure 8.7-D tracks 4 and 5). Collectively, these results indicate that ectopic, constitutive expression of p25 prevented transgene-induced silencing of the GFP transgene in the infiltrated region.

When the inducer of silencing was the replicating PVX-GFP-Δ25k-ΔCP construct the effects of p25 were more complex. In the (PVX-GFP-Δ25k-ΔCP+35S-25k) samples, at 2.5dpi, the levels of all high molecular weight RNAs were substantially higher than in the control (Figure 8.7-E, track 3 compared to track 1). This data indicate that p25 caused suppression of PTGS at this early time point. However, by 5dpi, even in the presence of p25, the target RNAs had all declined to lower levels than at 2.5dpi (Figure 8.7-E, tracks 2 and 4). The GFP mRNA from the transgene was masked by one of the viral subgenomic RNAs but it was clearly less abundant than in the mock-infiltrated tissue (Figure 8.7-E, track 4 compared to track 5, sgRNA2 and GFP RNA). This decline in the levels of target RNAs was observed in at least three independent experiments and indicates that, between 2.5 and 5dpi, local PTGS triggered by PVX-GFP-Δ25k-ΔCP had overcome the initial effect of p25.

The failure of p25 to prevent PTGS in PVX-GFP-Δ25k-ΔCP-infiltrated tissues was confirmed by the analysis of 25nt GFP RNAs. At 5dpi, these RNAs were 2.5 times more abundant in the presence of 35S-25k than with 35S-25k-ΔATG (Figure 8.7-F, tracks 2-3 compared to tracks 4-5), corresponding to the similar difference in PVX-GFP-Δ25k-ΔCP RNAs levels (Figure 8.7-E, track 2 compared to track 4). Thus, these 25nt GFP RNAs were likely generated primarily from replicating viral RNAs. In agreement with this idea, there was only a low level of 25nt RNAs in tissues that had been infiltrated with the non replicating PVX-GFP-Δrep-ΔCP construct (Figure 8.1) together with 35S-25k (Figure 8.7-F, track 1).

Collectively, these results indicate that the ectopically expressed p25 prevented systemic silencing irrespective of whether the inducer was a non replicating transgene construct (35S-GFP or PVX-GFP-Δrep-ΔCP) or replicating RNA (PVX-GFP-Δ25k-ΔCP, Figure 8.7-B). In contrast, local silencing was only suppressed by p25 if the inducer was a non replicating transgene construct (Figure 8.7-C, 5D and 5F, track 1). In this situation, the block on PTGS was associated with reduced accumulation of the 25nt GFP RNAs and, presumably, was targeted against either synthesis or processing of the precursor of these 25nt RNAs.

8.10. Discussion

8.10.1.Systemic signaling of PTGS and virus movement

The conventional models of virus movement involve opening of channels between cells: some viruses open plasmodesmata so that particles or viral ribonucleoproteins can move through; others produce tubules that extend from the surface of the infected cell and introduce virions into adjacent cells (Carrington et al., 1996). According to these models, the p25 protein of PVX has been characterized as a movement protein and was considered as a facilitator of channel gating. However, from the demonstration here, that replication of PVX induces systemic PTGS, it may be necessary to develop more refined models of virus movement (Carrington, 1999). These models will need to accommodate the anti-viral effect of systemic PTGS and the ability of viruses to suppress this process, as shown here with p25.

We anticipate that many viruses, like PVX, will have the potential to induce signaling of PTGS. It is possible that some of these viruses will resemble PVX in that they will prevent propagation of the silencing host response out of the infected cells. However, others may not have this capacity and would induce signaling of PTGS, as observed with PVX-rbcs-Δ25k-ΔCP and PVX-PDS-Δ25k-ΔCP (Figure 8.5). In these situations the PTGS signal could influence virus movement into and around cells that are several centimeters from the zone of infected cells.

A likely manifestation of virus movement influenced by systemic PTGS is in plants infected with nepo-, tobra-, caulimo- and other groups of virus that exhibit a pattern of symptom development referred to as recovery. Initially, these plants exhibit severe symptoms and the viruses are abundant in the infected tissues. Later, when the plants recover, the symptoms are mild and the viruses accumulate at lower levels. In these plants, we consider that the initially infected leaves could act as a source of signal that would potentiate resistance in the recovered leaves. Supporting this idea, it has been shown that there is RNA sequence-specific immunity against secondary infection in recovered leaves (Ratcliff et al., 1997; Ratcliff et al., 1999).

Signalling of PTGS may also play a role when viruses exhibit restricted movement on infected plants. For example, in *Arabidopsis* plants carrying the RTM1 and RTM2 loci (Whitham et al., 1999), tobacco etch virus (TEV) can replicate and move from cell-to-cell in the inoculated leaf but cannot spread systemically. It is striking that this effect is specific for TEV. Conceivably the products of RTM1 and RTM2 could affect the ability

of tobacco etch virus to prevent systemic spread of the sequence-specific silencing signal (Chisholm et al., 2000). Similarly, in the many examples where viruses replicate efficiently but are restricted to the initially infected cells (Matthews, 1991), it is possible that the silencing signal plays an important role. These subliminal infections could result if the signal moves ahead of the virus and prevents virus accumulation in cells that are adjacent to the site of initial infection.

Experiments involving double virus infection are also consistent with the widespread involvement of a PTGS signal (Atabekov and Taliansky, 1990). These experiments demonstrate that movement competent viruses could assist cell-to-cell trafficking of movement defective viruses even when they were from different viral genera. Previously, these findings were interpreted in terms of common mechanisms of virus trafficking. However, as the complementing pairs of viruses included combinations of tubule forming and plasmodesmatal gating viruses, a more

8.10.2. Two branches of the PTGS pathway

Transgene-mediated PTGS in *Arabidopsis* involves production of 25nt RNA and requires an RdRP homologue encoded by Sde1; in contrast, PTGS induced by some viruses appears to be independent of Sde1, although it also involves 25nt RNA (Dalmay et al., 2000). To explain these findings it was proposed that PTGS in plants is a branched variation of the pathway leading to RNA interference in *Drosophila*. This pathway involves processing of double stranded (ds)RNA into short 21-25nt RNAs that serve as the guide RNA for a sequence-specific nuclease (Zamore et al., 2000).

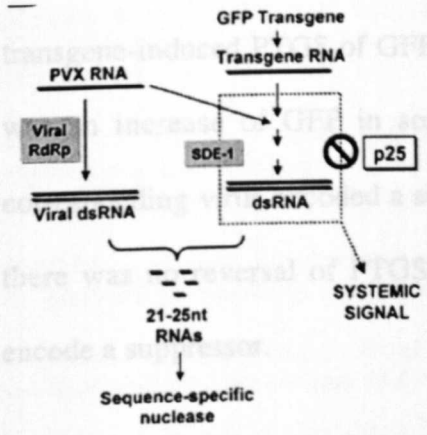
A previous suggestion was that, in plants, there are SDE1-dependent and SDE1-independent branches of the PTGS pathway (Dalmay et al., 2000). Both branches are dependent on synthesis of dsRNAs and converge at, or before, production of 25nt RNA. The dsRNA in the SDE1-independent branch would be produced through replication of the virus and would thus be dependent on the viral-encoded RdRp. In this model, the SDE1-dependent branch of the pathway is unaffected by viral RNA (Model 8.1).

To interpret the effects of p25 in terms of this model, we differentiate local and systemic PTGS. The local PTGS was suppressed by p25 if it was induced by a 35S-GFP transgene (Figure 8.7-C, 8.7-D) but not if the inducer was the replicating PVX-GFP-Δ25k-ΔCP (Figure 8.7-E, 8.7-F). Therefore, according to the model (Model 8.1), p25 would be a suppressor of the SDE1-dependent branch of the pathway. In contrast, systemic PTGS was suppressed by p25 irrespective of whether the inducer was the 35S-GFP transgene (Figure 8.7-B) or the replicating PVX-GFP-Δ25k-ΔCP (Figures 8.3 and 8.7-B). Thus, this effect of p25 on virus-induced systemic silencing is difficult to reconcile with the model presented (Model 8.1), in which the SDE1-dependent branch of the pathway is a transgene-specific process and is not affected by viral RNA.

In principle, these data could indicate that there are separate pathways, with multiple p25 targets, leading to local and systemic silencing. This possibility cannot be ruled out , although it seems unlikely because it requires that a virus-encoded protein would suppress the local PTGS induced by a transgene but not by a replicating virus. Instead,

we favour an alternative explanation in which p25 has a single target required for both local and systemic silencing. According to this explanation, the systemic signal would be produced in the SDE1-dependent branch of the pathway and, therefore, would be a precursor of the 25nt RNAs (Model 8.2).

This 'single target' explanation involves a refinement of the previous PTGS model (Model 8.2) in which the SDE1-dependent branch is not influenced by viruses. In the refined model, the virus-induced local PTGS would involve the SDE1-independent, p25-insensitive branch of the pathway, as previously. However, the SDE1-dependent, p25-sensitive branch is now recognised as being virus-induced (Figure 6B). As a result of this change, the model accommodates the finding that systemic signal production is influenced by PVX replication (Table 8.1, Figure 8.5) and is suppressed by p25.



Model 8.2. A refined model of PTGS based on the effect of p25 on local and systemic silencing. This model recognises participation of viral RNA in the SDE1-dependent branch. This branch may be involved in production of the systemic PTGS signal and is suppressed by the PVX-encoded p25 protein.

A further attraction of this refined model is that it resolves an apparent discrepancy between our results with Sde1 and those of Mourrain and colleagues (Mourrain et al., 2000) with Sgs2, which is identical to Sde1. In our analysis we found that mutation of Sde1/Sgs2 does not affect susceptibility to tobacco mosaic virus, tobacco rattle virus and

turnip crinkle virus (Dalmay et al., 2000) whereas Mourrain and colleagues found that mutations at this locus resulted in hypersusceptibility to cucumber mosaic virus. Presumably, the two sets of data differ because, of the viruses tested, CMV is the only one for which RNA accumulation is strongly limited by systemic PTGS. The other viruses are most likely limited by local PTGS which, as discussed above, would not be dependent on Sde1.

8.10.3. Dissection of PTGS using viral suppressors

The earlier characterization of viral suppressors involved infection of plants exhibiting transgene-induced PTGS of GFP (Chapters 6 and 7). With PVY and other viruses there was an increase of GFP in some or all of the infected tissues, indicating that the corresponding virus encoded a suppressor of PTGS. In contrast, in PVX-infected plants, there was no reversal of PTGS and we originally concluded that this virus does not encode a suppressor.

However, in the light of data presented here, in particular from the ectopic expression of p25 (Figure 8.7), it is clear that PVX does encode a suppressor of PTGS. It is likely that this property of PVX was not evident in the earlier experiments because the p25 protein of PVX and the other suppressors of PTGS, including HcPro of PVY, act on different stages in the gene silencing mechanism. The clearest indication that HcPro and p25 target different stages in gene silencing is from their differential ability to suppress virus-induced PTGS. The HcPro suppresses virus-induced PTGS of GFP whereas it is clear from the present and our previous studies that p25 does not (Chapter 3, Figures 8.2, 8.4-

A). Thus, according to the scheme in Model 8.2, HcPro should act on PTGS at some point after the convergence of the SDE1-dependent and SDE1-independent branches.

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CHAPTER 9

General discussion

Eukaryotic cells suppress foreign genetic elements through a nucleotide sequence-specific RNA turnover mechanism which, in this general discussion, will be referred to as "RNA silencing". This mechanism is remarkably efficient because, once activated by aberrant or foreign RNA molecules, it is primed against any cytoplasmic RNA species sharing sequence homology with the triggering molecules. RNA silencing can thus be envisioned as a form of immune system that operates at the nucleic acid level. RNA silencing was first discovered in transgenic petunia, in which expression of introduced transgenes and their homologous endogenous genes was co-ordinately suppressed (Napoli et al., 1990; van der Krol et al., 1990). Subsequently, it was shown that transgenes with no sequence homology to the plant genome could act as the trigger, as well as the target, of this suppression mechanism (Elmayan and Vaucheret, 1996). RNA silencing is also observed in animals, including pre-implanted mouse embryos (Wianny and Zernicka-Goetz, 2000), where it is experimentally activated by double-stranded (ds) RNA in a process referred to as "RNA interference" (RNAi). The mechanism of RNA silencing is progressively being unravelled and appears to be highly conserved across kingdoms. This is evidenced by recent genetic and biochemical studies conducted in *Caenorhabditis elegans, Drosophila*, as well as in *Neurospora, Chlamydomonas* and *Arabidopsis* species (reviewed in (Hammond et al., 2001)). In animals, a natural role for RNA silencing is in the control of transposable elements (Ketting et al., 1999; Tabara et

al., 1999). In higher plants, where more than 90% of viruses have an RNA genome, the RNA-based nature, high efficiency and *trans*-acting property of RNA silencing make it a potent defence mechanism against viruses. Presumably, nuclear-encoded transgenes activate RNA silencing in plants because the corresponding transcript is perceived by the cell as if it were a virus. By incorporating the results presented in this thesis with some of the recent mechanistic advances made across kingdoms, this chapter will provide a general view on our current understanding of the antiviral function of RNA silencing in plants. The potential role of RNA silencing in plant development and physiology will also be discussed.

9.1 A generalised response to viruses

For decades, the prophylactic inoculation of attenuated viral strains has been used to control viral diseases in plants. The rationale for this form of vaccination, called 'cross-protection', came from classic observations that many infections in plants prevent the secondary accumulation of viral strains that are closely related to the primary-infecting virus. For several plant viruses, the mechanism underlying cross-protection -or at least part of it- is a post-transcriptional and RNA-mediated process that targets the secondary-challenged virus in a nucleotide-sequence-specific manner (Covey et al., 1997; Ratcliff et al., 1997; Ratcliff et al., 1999). Using the persistent and symptomatic infection of potato virus X (PVX) as a general model, it was further established that this defence response is functionally equivalent to RNA silencing because it is based on a *trans*-acting and sequence-specific mechanism that degrades virus-homologous, single-stranded RNAs (Ratcliff et al., 1999).

These findings about cross-protection were consistent with the observations, described in chapter 3, with plants infected with PVX vectors that carried exon fragments of host nuclear genes. In these experiments, infected tissues developed symptoms that were phenocopies of mutations in the corresponding nuclear genes and were caused by a decline in the host mRNA levels. In effect, the RNA silencing normally activated by the virus targeted both the viral and the endogenous RNAs because the genetically modified PVX had similarity to a plant gene. Significantly, this phenomenon, which we termed virus-induced gene silencing (VIGS), has been since reported for a variety of virus–host combinations (Table 1). These observations imply that each of these viruses activates RNA silencing in natural infection and, therefore, provide compelling evidence for a generalised plant defence response to RNA as well as DNA viruses (Table 1). As VIGS operates in the primarily infected cells (Chapter 3, Figure 3.2-B), it is likely that the host RNA silencing response contributes to lessen the impact of viral diseases at early stages of infection. This defence system is remarkable in its ability to adapt to – potentially – any virus, because its specificity is not genetically programmed by the host but, instead, is dictated by the genome sequence of the viral intruder itself.

9.2 The systemic arm of RNA silencing

9.2.1 Systemic silencing in transgenic plants

A second remarkable feature of RNA silencing is that it can propagate to distant parts of the plant *via* a sequence-specific signal. The experiments reported in chapters 4 and 5

demonstrated the existence of systemic silencing in transgenic plants. Independent work, based on graftings, confirmed that production of a systemic signal is also a feature associated with other transgene silencing systems. Signaling in these various systems had a number of shared properties, the most striking of it being the capacity of perpetuation. This feature was dramatically illustrated in bombardment experiments, in which ectopic DNA copies were used to trigger systemic silencing of a stably integrated and initially highly expressed GFP transgene (Chapter 5). Biolistic introduction of the ectopic DNA occured in a few cells only, yet eventually the whole plant exhibited the silencing phenotype, long after senescence of the bombarded leaf. This provides direct evidence that the silencing signal is amplified, probably in each recipient cell.

It is likely that this amplification process has two distinct components. The first component is the acquisition, by recipient cells, of the property to maintain silencing independently of signal input. We can refer to this property as "maintenance". The concept of maintenance was initially evoked in chapter 3 to explain the persistence of VIGS targeted against a GFP transgene in the absence of the PVX-GFP inducer. We also discussed in chapter 5 the potential mechanistic link between initiation and maintenance steps of systemic silencing. This link was not clear from the present work because the GFP loci in the individual transgenic lines 16c, 17b, Y, and 8 were equally responsive to a bombarded DNA trigger of systemic silencing. However, in another experimental system based on silencing of the nitrate reductase (NR) gene, which is manifested as chlorosis, there were several transgenic lines that failed to produce systemic silencing

following bombardment of homologous DNA (Palauqui and Balzergue, 1999). These plants could still perceive the silencing signal because when they were grafted onto silenced transgenic rootstocks, they became progressively chlorotic, as a result of NR cosuppression (Palauqui and Vaucheret, 1998). However, these scions failed to maintain silencing when they were detached from the rootstocks. In contrast, if similar experiments were performed using scions from transgenic lines in which systemic silencing could be triggered by bombardment, silencing of NR was maintained after degrafting (Palauqui and Vaucheret, 1998). Thus, maintenance and initiation of systemic silencing seem to have similar requirements and may actually constitute a single step of the mechanism. Maintenance, seen at the whole plant level, could result from iterated initiation events that would therefore contribute to amplify the silencing response. As proposed in chapters 3 and 5, one of the requirements for maintenance may be the production of a particular transgene RNA (the so-called 'aberrant RNA') specifically required for systemic silencing and maintenance. In transgenic plants, production of this RNA would be conditioned by the capacity of certain transgene loci to undergo an epigenetic change.

The second component in the amplification process is the relay of RNA turnover induced by the silencing signal. As opposed to maintenance, this step does not require the presence of a "competent" transgene to occur. For instance, nontransgenic tobacco mutant plants that over-accumulate the NR mRNA become silenced when grafted onto transgenic rootstocks that produce a NR silencing signal (Palauqui and Vaucheret, 1998). However, the extent of RNA degradation activated into the scions seems to be

conditioned by the level of target mRNA. Hence, wildtype tobacco plants, with normal NR mRNA levels, do not show chlorosis when grafted onto the co-suppressed NR transgenic stocks (Palauqui and Vaucheret, 1998). This result is intriguing because in both experiments the amount of silencing signal received by the scions is most likely the same. To account for the difference, one can propose the existence of a maintenance-independent relay-amplification mechanism. The efficiency of this relay would be directly related to the abundance of target mRNA and would determine the extent of local silencing in areas that receive the systemic signal. An implication of this hypothesis is that maintenance and relay-amplification might be ensured by distinct signalling molecules. This issue will be discussed in section 9.5.2.2.

9.2.2 Systemic silencing in non-transgenic plants

The relevance of systemic silencing during virus infection was addressed in Chapter 8. Both the timing and routes of transgene-induced systemic silencing are strikingly reminiscent of a virus infection and this prompted speculation that the silencing signal could be part of the natural RNA silencing response to viruses. However, investigation of an antiviral silencing signal in non-transgenic plants was experimentally challenging. First it required uncoupling movement of the signal from that of the virus. Second, the experiment had to be designed so that propagation of the virus-induced signal could be somehow monitored *in planta*. To overcome these difficulties, RNA silencing was activated in upper leaves of *N. benthamiana* plants by inoculating lower leaves with movement-defective mutants of PVX containing fragments of endogenous plant genes. It was reasoned that the effect of a viral-induced silencing signal would be manifested as

host-gene-specific silencing phenotypes developing away from the leaf where the modified virus was introduced. This experimental system provided an indirect indication that replication of PVX was required to trigger a systemic silencing signal in wild type plants. Moreover, it was shown that the signalling molecule could prevent accumulation of transiently expressed, virus-specific RNA, demonstrating its antiviral nature.

However, in these experiments, the signalling was only transient and appeared as a "wave". This transience can be explained by the fact that the target RNAs were from endogenous genes that would be unable to ensure the maintenance phase of the process (chapter 3). In addition, the transient replication of the engineered PVX confined into the lower inoculated leaf was probably the only source of signal. Under those conditions, the amount of systemic signal received in the leaves was therefore probably limited. These combined factors likely contributed to the restriction of the silencing in or near the veins of a few systemic leaves. It is not known at present if this pattern of signalling is representative of the situation that would be observed in the course of a real virus infection, in which the antiviral signal would move with the pathogen. In theory, a maintenance-like situation could be achieved if the virus produces an RNA that is qualitatively similar to the aberrant RNA evoked above to account for maintenance of transgene-induced systemic silencing. Even in the absence of maintenance, the high levels of viral RNA could be sufficient to provide efficient relay amplification, as shown in the NR over-accumulating, non-transgenic tobacco plants.

Although direct evidence for signalling in normal virus infection is still lacking, the discovery of a systemic component of RNA silencing indicates that it holds key features of an elaborate immune system in plants: it is adaptive, mobile and specific.

9.3 Viruses fight back

9.3.1 Viral-encoded suppressors of RNA silencing

The simple observation that viruses infect plants at all indicates that they have somehow evolved to avoid or suppress the host RNA silencing response. This idea was first prompted by the demonstration that co-inoculation of a normally mild virus with potyviruses leads to an extreme accentuation of symptoms caused by high accumulation of the non-potyviral partner (Pruss et al., 1997). It was shown that this synergism results from suppression, by the Hc-protease (HcPro) of potyviruses, of a host defence mechanism that normally restricts multiplication of the second virus (Pruss et al., 1997). Three independent studies, including the one described in chapter 6 further demonstrated that expression of HcPro, either transgenically or from a virus vector, was sufficient to overcome the antiviral host defence and simultaneously inhibit transgene-induced and virus-induced RNA silencing (Anandalakshmi et al., 1998; Kasschau and Carrington, 1998). From these results, it was proposed that the host defence suppressed by HcPro is akin to RNA silencing.

The link between disease severity and silencing suppression was further strengthened by the finding that several proteins that have been previously identified as required for viral pathogenicity suppress transgene-induced silencing (Chapters 6, 7). It should be noted,

however, that two mutant forms of the 2b protein retain the silencing suppression activity of the wild-type protein but do not cause enhanced virulence when expressed from the PVX vector. This finding indicates that suppression of silencing is important but not sufficient for virulence determination by the 2b protein.

A survey of more than 15 viruses confirmed that suppression of RNA silencing is a general property of plant viruses, and it is likely that many additional suppressor proteins will be identified in the near future (see section 9.3.5.2). In chapter 8, the PVX-encoded p25 movement protein was characterised as a novel type of silencing suppressor that seems to specifically prevent systemic signalling of RNA silencing.

9.3.2 Diversity and evolution of silencing suppressors

A striking aspect of the virus-encoded suppressors of silencing is their high diversity in sequence and structure. These proteins are also functionally diverse, as revealed by their distinct effects in overcoming transgene-induced and virus-induced silencing (Chapters 6-8). To some extent, this diversity can be explained by the fact that silencing suppression may have evolved as an additional feature of an existing protein. In addition, these factors may have evolved in concert with their host protein interactors. If that is the case, the diversity of suppressor proteins will reflect host-virus interactions rather than functional differences.

This idea of co-evolution between plant components of RNA silencing and the virus-encoded suppressors is consistent with experimental evidence. For instance, the study of

various potexviruses in *Nicotiana benthamiana* revealed an extreme variability in the suppression of silencing caused by highly related members of this single virus group (Chapter 7). Also, the 2b protein of tomato aspermy virus (TAV) in *N. benthamiana* is a more efficient suppressor than the related 2b protein of cucumber mosaic virus (CMV), even if both factors are expressed at similar levels from the PVX vector (Li et al., 1999). These findings may reflect a variation in the strength of the interaction between distinct forms of the suppressor and the host target to which they are adapted. Also in agreement with a co-evolution, it has been shown that transgenic expression of the tobacco etch potyvirus (TEV) HcPro suppresses RNA silencing in tobacco but not in *Arabidopsis*, a non-host species of TEV. In contrast, the HcPro of turnip mosaic virus, which infects *Arabidopsis*, suppresses transgene silencing in this species (K. Kaschau and J. Carrington, pers. commun.).

Having identified suppressors of gene silencing, the next logical step is their use to characterise the host target proteins, following a genetic approach in *Arabidopsis* for instance (See section 9.5.4.2). However, the consequences of co-evolution of host factors with viral suppressors could complicate this task because most of these proteins have been isolated from viruses for which *Arabidopsis* is not a host. T-DNA constructs containing expression cassettes of most suppressors have been engineered and transformed into *Arabidopsis* lines that exhibit PTGS. The effect of each protein is currently being investigated.

9.3.3 Suppression and other viral strategies to counteract RNA silencing

It should be stressed that suppression of PTGS may be one of the many ways viruses counteract silencing in plants. For instance, it is conceivable that the compartmentalisation of replication (for instance in membranes) or the capacity to move quickly from infected to uninfected cells contribute significantly to defend the pathogen against RNA silencing. However, in the current state of our knowledge and technical skills, the contribution of such parameters is difficult to appreciate experimentally.

In investigating the connection between RNA silencing and viruses, it is also worth considering that viruses are obligate parasites and hence, that silencing might somehow be beneficial to the virus by preventing the detrimental effects of over-accumulation. An extreme illustration of this idea might be provided by an unusual type of RNA silencing response to viruses, called recovery. Recovery is observed in plants that are initially highly symptomatic but that later appear healthy. The asymptomatic tissues have much lower levels of virus than the symptomatic parts of the plant, although the pathogen is never eliminated completely. Because recovery-inducing viruses do not usually encode suppressors of silencing, this peculiar interaction could represent an exacerbated plant silencing response.

However, Franck Ratcliff pointed out in his thesis the striking correlation between the ability of a virus to cause recovery and to infect meristems, from which reproductive cells form. This is true for nepoviruses, alfalfa mosaic virus, tobacco rattle virus and also for avocado sunblotch viroid. In these examples, pollen transmission of the pathogen is an

indicator of meristem entry. Most plant viruses are normally excluded from meristematic areas and although the biological basis for this phenomenon has not yet been elucidated, it is possible that meristem exclusion is mediated by RNA silencing. Recovery-inducing viruses could represent a class of viruses that exhibit a high tolerance to silencing, so that they would not need to produce suppressor proteins and they would only be partially inhibited in apexes, whereas non-recovery inducing viruses presumably would be completely eliminated in those tissues.

Thus, recovery could be envisaged as a viral adaptation allowing pollen transmission. By tolerating rather than suppressing RNA silencing, recovery-inducing viruses could preserve the integrity of their host, ensuring flowering and thereby horizontal propagation. Another strong selection against silencing suppression -as opposed to tolerance- by these viruses could be the possible role of RNA silencing in genome defence against transposable elements, as shown in animals(Tabara et al., 1999) (Ketting et al., 1999). Indeed, suppression of silencing in meristems would result in highly detrimental and heritable genetic instability of the viral host.

9.3.4 Suppression of RNA silencing and virus movement

With the exception of AC2, all the suppressors characterised to date have a role in viral long-distance movement. From this observation, it can be inferred that these viral movement proteins might not simply mediate the transport of viral RNA between cells or through sieve elements, as proposed in conventional models for virus movement. It could be that these proteins have an indirect effect on virus movement mediated by silencing

suppression. The suppression could act at the intracellular level allowing sufficient accumulation of the viral genome for spread to occur from one cell to another. Virus movement could also result from inhibition of silencing in cells that are about to be invaded, with the silencing suppressor acting non-cell-autonomously. Non cell autonomous action of the suppressor would require that the protein is able to traffic between cells. A third possibility is that the suppressor prevents the silencing signal moving from an infected to an uninfected cell, forewarning of the virus infection, as proposed for the p25 protein of PVX. The potyviral HcPro might provide an example of a silencing suppressor that possesses all of the properties listed above. Hence, HcPro mediates its own movement between cells (Rojas et al., 1997) and the central domain of the protein, which is involved in synergism (Ming Shi et al., 1997), is also required for genome amplification in single cells as well as in long distance movement in whole tobacco plants (Kasschau et al., 1997).

9.3.5 Silencing suppression by viruses and its implication for genetic engineering and biotechnological applications

9.3.5.1 Silencing suppressors as tools for protein over-expression

In many cases, it is desirable to express high levels of a foreign gene or to overexpress an endogenous gene. However, high level expression of transgenes is often impeded by RNA silencing. Suppressors of silencing can be used to counter the effects of PTGS and thus have significant potential to improve yields in biotechnological applications. For instance, it has been shown that introduction (by stable transformation or by cross) of the TEV HcPro into silenced tobacco lines give consistent, high level expression of previously silenced transgenes (Kasschau and Carrington, 1998). However, there could be adverse outcomes in the use of suppressor-expressing plants in the field, as these transgenic plants would probably be hypersusceptible to many viruses (Pruss et al., 1997).

I have reported in chapter 8 that the PVX-encoded p25 protein could be used transiently to prevent initiation of local silencing in the *Agrobacterium*-infiltrated patch of GFP transgenic plants. Interestingly, not only did p25 prevent silencing of the stably integrated transgene, it also caused enhanced expression of the ectopic GFP delivered in the T-DNA. This effect was evident in Northern analysis, but also through inspection of the GFP produced in the infiltrated patch. The p25-treated samples appeared much brighter than similar patches treated with the non-translatable form of p25. This prompted me to

investigate the phenomenon further, as the result suggested that *Agrobacterium*-mediated transient expression *per se* caused PTGS. A direct test of this hypothesis was to repeat the experiments in nontransgenic *N.benthamiana* and to measure the effect of various silencing suppressors (cloned individually in T-DNA expression cassettes) on accumulation of the transiently expressed GFP. The accumulation of 21-23nt RNA, diagnostic of PTGS, was also monitored. The results of these experiments indicated that a large amount of GFP small RNA is consistently produced shortly after infiltration and that it accumulates for up to 10 days post-infiltration (O.V and A. Hamilton, data not shown). Interestingly, we found that some silencing suppressors could inhibit accumulation of the small RNA, and accordingly, caused an increase and stabilisation of the ectopically expressed GFP mRNA.

The most dramatic results were obtained with the p19 protein of tomato bushy stunt virus. The images and western-blot in Figure 9.1 illustrate the compared effect of transient expression of p19 and HcPro on the accumulation of GFP produced from the epiGFP construct (Chapter 4, Figure 4.1) in infiltrated leaves of wild type *N.benthamiana*. It should be noted that the levels of GFP synthesised transiently in combination with p19 were considerably higher than those produced by several individual stable GFP transformants that were considered as "high expressor lines" (lines 16c and 8). This enhancement effect was not a peculiarity of GFP because it was subsequently observed with many constructs expressed transiently. For instance, Figure 9.1-F shows enhanced accumulation of a truncated form of a tomato calcium-dependent

protein kinase (CDPK) that was transiently expressed in combination with p19 in leaves of *N.benthamiana* (Tina Romeis, unpublished data).

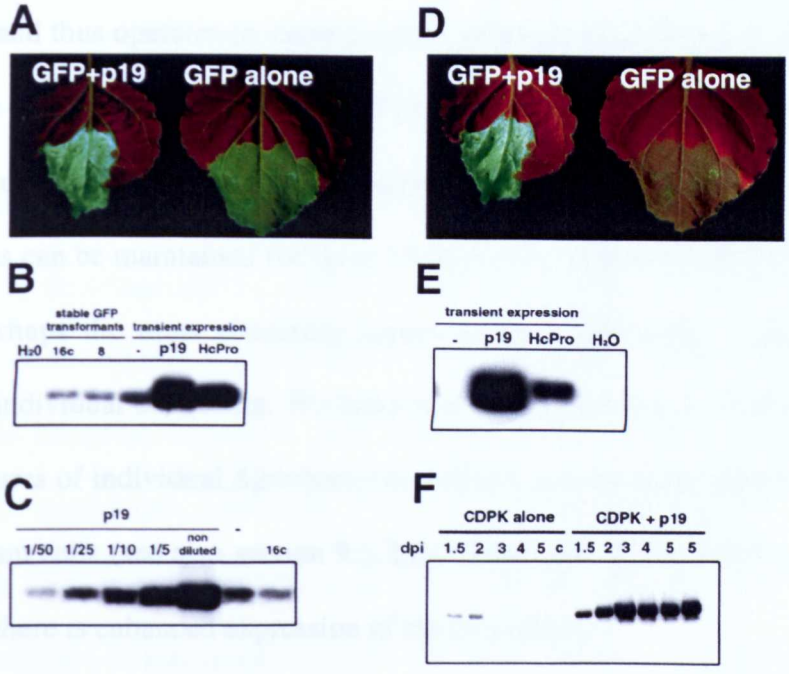

Figure 9.1. *Agrobacterium*-mediated transient expression is enhanced by p19. (**A**) Phenotypes of epiGFP transient expression in the presence (left) or absence (right) of p19 in leaves of wild type *N.benthamiana*. Detached leaves were imaged under UV illumination at 5dpi. (**B**) Comparison of the effect of p19 and HcPro on epiGFP accumulation at 5dpi. The levels of epiGFP were assessed by western blot analysis, using a GFP antibody. Two GFP stable transformants (lines 16c and 8) were also used as controls. Note that transient expression without the silencing suppressors (track 4) already causes a 2 to 3 fold increase in GFP levels, compared to the amount of protein produced in the stable transformants 16c and 8. Equal protein loading was confirmed by membrane staining (not shown). (**C**) The enhancement caused by p19 was quantified by diluting total proteins extracted from the p19-treated sample (track 5 in (B)) into total proteins extracted from a non-treated leaf. The increase in epiGFP synthesis caused by p19 is about 25-50 times than the 16c stable transformant. (**D**) Long-lasting effect of p19. The detached leaves shown in (A) were kept on humid WHATMAN 3MM paper and imaged again at 12 dpi under UV illumination. The enhancement in epiGFP levels caused by p19 is still clearly observed (left), whereas epiGFP in the control experiment (right) is hardly detected (the dark brown colour is from slight chlorosis of the *Agrobacterium*-infiltrated area). (**E**) Western blot analysis illustrating the compared effect of p19 and HcPro on epiGFP accumulation at 12 dpi. EpiGFP is below the detection limit in the control sample (track 1). Equal protein loading was confirmed by membrane staining (not shown). (**F**) The effect of p19 is not peculiar to epiGFP. Time course analysis of the accumulation of a truncated form of a tomato calcium-dependent protein kinase (CDPK). The truncated CDPK cDNA was cloned under the 35S promoter and subsequently mobilised in the pBin19 T-DNA. The construct was then used in *Agrobacterium*-mediated transient expression assays with or without co-expression of p19. Accumulation of CDPK was measured by Western blot analysis using a CDPK antibody. The enhancement caused by p19 at 3dpi is >1000 fold. Equal protein loading was confirmed by membrane staining (not shown). P19 is now routinely used in the laboratory for a variety of applications for which high levels of a given protein are required. So far p19 proved to be effective when used with a broad variety of proteins.

This transient assay for high level of protein expression has a number of advantages over stable transformation. Firstly, it is very rapid: for any cloned cDNA, enhanced levels of protein can be obtained within a few days. Secondly, the system does not rely on stable transformation, and thus operates on nontransgenic plant material. Third, it can be easily scaled-up, as the only requirement is a form of high-throughput infiltration procedure (i.e. large-scale vaccum leaf infiltrator) and a humid chamber to maintain detached leaves (infiltrated leaves can be maintained for up to 15 days on humid WHATMAN paper, see Figure 9.1). Perhaps the most attracting aspect of this technology is that it allows combination of individual constructs. We have now confirmed that 3 T-DNAs provided by a mix of cultures of individual *Agrobacterium* strains can be co-transferred in a high proportion of plant cells (see also section 9.5.2.2). When one of the T-DNAs expresses the p19 protein, there is enhanced expression of the two others.

Practically, this means that complicated pathways for engineering valuable proteins could be reconstituted transiently and that the synthesis of the end-product could be enhanced. For instance, using this transient assay system, we have confirmed that co-expression of a viral protease and a target protein containing the appropriate cleavage site, in combination with the p19 protein, causes a strong enhancement in the accumulation of the expected cleaved product (Pere Mestre and O.V. data not shown). Finally, this technology can also be used with viral expression vectors, such as the PVX vector, which, as shown in this thesis, is normally a strong inducer of PTGS. Indeed, co-infiltration of the PVX-GFP-ΔCP strain of *Agrobacterium* (Chapter 8) with a strain carrying a p19 expression cassette leads to a strong enhancement of GFP synthesis

through viral replication. This result is interesting, as the PVX coat-protein can be fused to foreign proteins in such a way that it still allows viral encapsidation. Purification of viral particles could thus be used as a means to enhance or facilitate purification of the protein of interest.

9.3.5.2 Identification of new silencing suppressors

The transient assay described above could also be used as a simple and rapid method for identification of novel silencing suppressors. Because it does not rely on transgenic plants, the system could be adapted to potentially any plant in which *Agrobacterium*-mediated transient expression is possible. First, a "candidate gene" approach could be used, whereby a viral protein with features of silencing suppressor (i.e. a protein previously characterised as a pathogenicity determinant) would be co-expressed with the epiGFP construct. Enhanced green fluorescence in the infiltrated tissue would indicate that the protein is a potential silencing suppressor. This strategy has been used successfully to confirm that the 15 kDa protein of peanut clump pecluvirus and the P0 protein of beet western yellow vein luteovirus are silencing suppressors in *N.benthamiana* (P. Dunoyer, S. Pfeiffer, O. Voinnet and Ken Richards, data not shown). In a forward approach, a cDNA library representing many individual viral-encoded proteins (isolated from many types of plant viruses) could be used in a high throughput screen for enhanced green fluorescence.

9.3.5.3 Silencing suppression as a threat to the exploitation of transgenic plants

The fact that many viruses encode suppressors of PTGS (chapter 7) represents a potential risk for the exploitation of transgenic plants in which gene silencing is responsible for an ameliorated trait. In the case of virus-resistant crop exhibiting HDR (see Chapter 1), for instance, it is conceivable that infection by heterologous viruses producing suppressors of PTGS would alleviate the resistance.

9.4 Mechanisms of RNA silencing as a response to virus infection

9.4.1 Initiation: a central role for double-stranded RNA?

Transgenic plants expressing single-stranded (ss) sense RNA normally exhibit RNA silencing in any 5-20% of the independent lines. However, those engineered to produce dsRNA cause a higher incidence of RNA silencing (Chuang and Meyerowitz, 2000; Smith et al., 2000). This finding is consistent with the demonstration that dsRNA is sufficient to trigger RNAi in animals (Fire et al., 1998). Moreover, most of the transgenic lines that express replicating PVX RNA (in which dsRNA replication intermediates are formed) also exhibit RNA silencing (Angell and Baulcombe, 1999).

These collective observations support suggestions that cytoplasmic dsRNA produced during replication of RNA viruses (i.e. PVX) could be the trigger of silencing in infected cells. However, this idea has not yet received direct experimental support and it remains possible that the RNA silencing trigger could be some particular ssRNA species (for instance, subgenomic RNAs that are only produced during replication). These RNAs could have secondary structures or other qualitative features that would make them easily perceived by the host silencing machinery.

If double-stranded RNA is the initiator of RNA silencing from DNA viruses, it would have to be produced fortuitously during genome transcription/amplification. For instance, bi-directional transcription of the geminivirus circular DNA could lead to synthesis of complementary sense and antisense transcripts that could anneal to form dsRNA (Mullineaux et al., 1992). In caulimovirus-infected cells, dsRNA could result from

$tRNA_{met}$ priming of the 35S RNA (Matthews, 1991). Alternatively, dsRNA could be produced by a host-encoded RNA-directed-RNA-polymerase (RdRp) using viral ssRNA as template, as discussed later. It is also possible that the trigger of silencing in DNA virus-infected tissues is not dsRNA. Following an analysis of RNAi mutants in *C. elegans* (Grishok et al., 2000), it has been suggested that a non-dsRNA stimulus of RNA silencing could cause suppression of transposon mobilisation in this organism (Sharp and Zamore, 2000).

9.4.2 21–23nt RNA as specificity determinant of RNA silencing in virus-infected cells

As mentioned in chapter 8, a discrete species of low molecular weight antisense RNA (approximately 21–23 nucleotides (21–23nt) long), with sequence homology to the targeted transcript, accumulates consistently in plants exhibiting RNA silencing (Hamilton and Baulcombe, 1999). Similar sized RNA molecules in animals undergoing RNAi co-purify with a sequence-specific nuclease activity that, in *Drosophila* S2 cells, is part of a muti-subunit complex named RNA-induced silencing complex (RISC) (Hammond et al., 2000). Recently, it was confirmed that synthetic 21–23nt-long RNA is necessary and sufficient to direct the RISC-mediated degradation of homologous, single-stranded transcripts in *Drosophila* embryo lysates (Elbashir et al., 2001). The current view is that these low molecular weight RNAs serve as guide RNA for the RISC complex. In plants, PVX-specific 21–23nt RNA is detected in PVX-infected tissues (Hamilton and Baulcombe, 1999) (see also chapter 8). 23nt is also the minimal length of homology required for VIGS of a GFP transgene RNA by the PVX vector (Thomas et al.,

2001). Taken together, these findings indicate that 21–23nt RNAs are probably the specificity determinants of RNA silencing in virus-infected cells .

9.4.3 Processing of dsRNA into 21–23nt RNA and degradation of ssRNA: separable steps of the core mechanism of RNA silencing

In plants as in animals, 21–23nt RNA of sense polarity accumulates at the same abundance as the antisense species (Elbashir et al., 2001; Hamilton and Baulcombe, 1999). This observation led to the suggestion that dsRNA, a trigger of RNA silencing, is a precursor of these molecules. Recently, the work of Bernstein and colleagues provided direct support to this hypothesis. It was shown that a *Drosophila* RNAse-III-related protein, Dicer, processes dsRNA but not ssRNA into 21–23nt RNA *in vitro* (Bernstein et al., 2001). Depletion of Dicer *in vivo* decreases the ability of *Drosophila* cells to carry out RNAi. Importantly, Dicer activity could be separated from the RISC complex that degrades ssRNAs (Bernstein et al., 2001). These findings allow the conceptual dissection of what might form the core mechanism of RNA silencing into three sequential steps (Fig. 9.2): (1) dsRNA processing into 21–23nt RNA; (2) incorporation of the 21–23nt RNA into the RISC complex; and (3) RISC-mediated ssRNA degradation. *In vivo*, RISC and Dicer could interact with each other through a protein motif known as PAZ, which is present in the amino acid sequence of Dicer and of AGO2, a component of RISC. AGO2 belongs to the ARGONAUTE/ZWILLE/PINHEAD/PIWI/STING class of proteins, which is related to translation initiation factor eIF2C (Baulcombe, 2001; Bernstein et al., 2001). To accommodate the high potency of RNAi (small amount of dsRNA can cause

degradation of highly expressed mRNA), most models assume that the RNA degradation step mediated by RISC is a catalytic process.

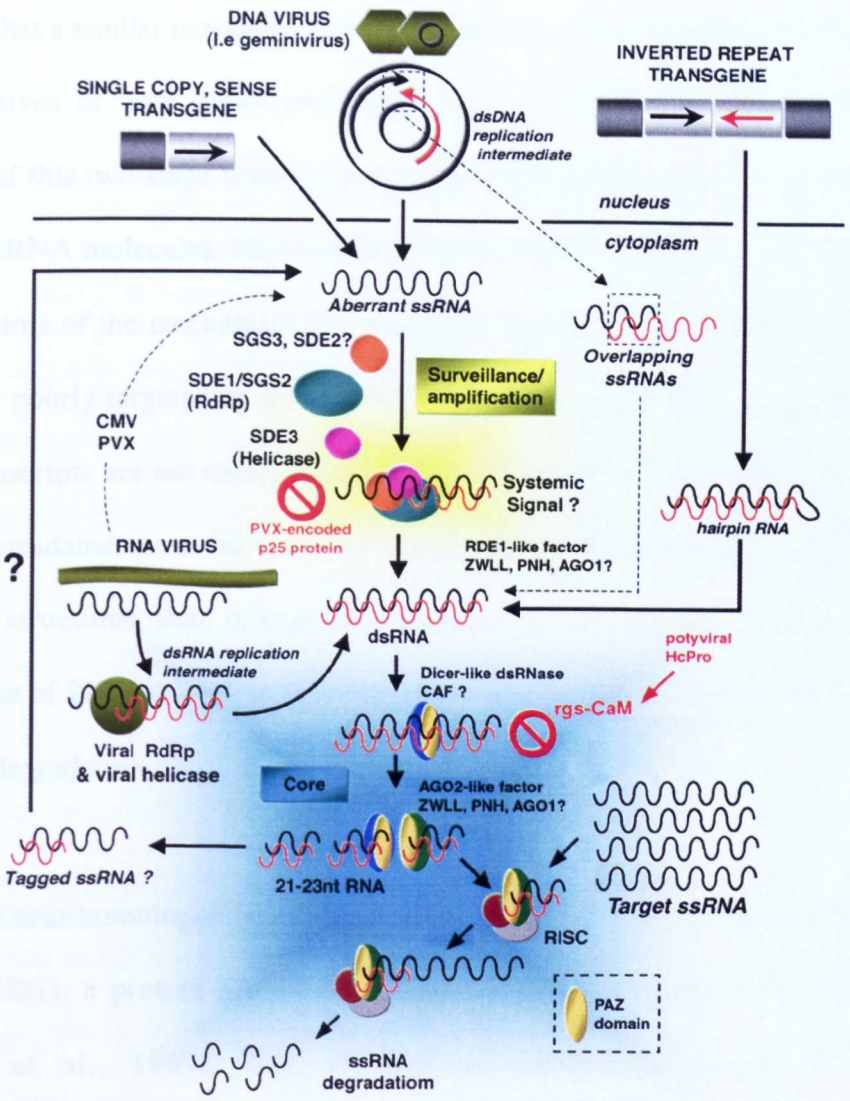

Figure 9.2 A model for RNA silencing as a response to virus infection in plants. The model accommodates the data generated from genetic screens in *Arabidopsis* identifying factors required for silencing of single copy sense transgenes. Sense transgenes are believed to be silenced through a mechanism that detects and converts aberrant RNA into double-stranded (ds)RNA (SDE/SGS surveillance-amplification system). Transgenes with inverted or rearranged structures might produce directly dsRNA through intramolecular base-pairing and thus activate silencing independently of the SDE/SGS system. This might also be the default pathway of silencing triggered by RNA viruses, which produce dsRNA as part as their replication cycle. Some RNA viruses, such as CMV or PVX, could also produce aberrant RNA that are processed in the SDE/SGS branch. Combined analyses of the PVX-encoded p25 protein and the potyviral HcPro suggest that the systemic signal might be produced in the SDE/SGS branch and could be a precursor molecule of the 21–23nt RNA. The various possible branches through which silencing is activated are thought to converge at the accumulation of dsRNA. This molecule is then processed by the core mechanism of silencing in a two-stage mechanism involving: (1) 21–23nt RNA synthesis; and (2) incorporation of the 21–23nt RNA into a nuclease complex that degrades ssRNA in a sequence specific manner. The position of AGO1, which is required for RNA silencing in *Arabidopsis,* is not yet defined (see text).

It is likely that a similar mechanism also constitutes the core of RNA silencing in plants, where relatives of both Dicer and AGO2 exist. In terms of antiviral defence, the advantage of this two-stage process is that a large response could be mounted against a few viral dsRNA molecules. However, it is conceivable that some viruses, despite being good activators of the mechanism (by producing high amounts of dsRNA for instance), are actually poorly targeted by the ssRNA degradation machinery because their genome or their transcripts are not easily accessible to it. This could occur if progeny RNA are rapidly encapsidated, or, as for viroids, if the genome of the pathogen possesses extensive secondary structures that cannot be resolved by the nuclease. This means that accumulation of 21-23nt RNA in infected cells is not a complete indicator of the extent of viral RNA degradation.

A possible Dicer homologue in *Arabidopsis* is Carpel Factory (CAF, also referred to as SIN1 or SUS1), a protein previously characterised as a regulator of stem cell fates (Jacobsen et al., 1999). The product of *AGO1*, a member of the plant *ARGONAUTE/ZWILLE/PINHEAD* multigene family, is also required for stem cell identity (Bohmert et al., 1998; Lynn et al., 1999; Moussian et al., 1998) and was recently shown to be necessary for transgene-induced RNA silencing in *Arabidopsis* (Fagard et al., 2000). AGO1 is thus a possible homologue of the *Drosophila* RISC-associated AGO2, although the exact position of AGO1 in the plant silencing pathway remains to be determined. It is possible that AGO1 acts upstream and independently of RISC, as previously proposed for RDE1, an ARGONAUTE-like protein of *C. elegans*. RDE1 is

indeed necessary for RNAi initiation, although the *RDE1* gene appears to be dispensable for the inherited effect of silencing in progeny worms (Grishok et al., 2000).

9.4.4 Transgene-induced versus virus-induced RNA silencing

9.4.4.1 SDE1 and SDE3 are likely required for dsRNA synthesis and are dispensable for silencing induced by many RNA viruses

Based on forward genetic screens, several loci that are required for transgene-induced RNA silencing in *Arabidopsis* have been identified (Dalmay et al., 2000; Elmayan et al., 1998) and three of the corresponding genes have recently been cloned. *SDE1/SGS2* encodes a putative RNA-dependent RNA-polymerase (RdRp) (Dalmay et al., 2000; Mourrain et al., 2000) and is thus a plant homologue of *QDE-1*, which is required for RNA silencing induced by sense transgenes in *Neurospora* (Cogoni and Macino, 1999). *SGS3* encodes a protein of unknown function (Mourrain et al., 2000) and the product of *SDE3* is similar to RNA helicase-like proteins found in mouse, *Drosophila* and humans (Dalmay et al., 2001). Although the *Arabidopsis sde1/sgs2, sgs3* and *sde3* mutants are all defective in RNA silencing mediated by sense transgenes, they are as susceptible as the wildtype *Arabidopsis* to many RNA viruses (Dalmay et al., 2000; Dalmay et al., 2001; Mourrain et al., 2000). As most of these viruses encode suppressors of silencing, this observation was initially interpreted as a consequence of silencing inhibition in both wild type and mutant plants, such that there was no discernible effect of the mutations (Mourrain et al., 2000). However, the levels of tobacco rattle virus (TRV), which does not suppress silencing in the parental line, are as high in the *sde1/sgs2* and *sde3* mutant as in wild type *Arabidopsis* (Dalmay et al., 2000; Dalmay et al., 2001).

The lack of effect of the three *Arabidopsis* mutants on accumulation of RNA viruses, regardless of their capacity to suppress silencing, indicates that the corresponding host proteins are not necessary for virus-induced gene silencing. This finding supports a model in which SDE1/SGS2, SDE3 and possibly SGS3 are required to synthesise a dsRNA initiator of silencing (Fig. 9.2). According to this idea, in virus-infected cells, the dsRNA trigger of silencing would be produced by viral-encoded RdRps and RNA helicases under the form of replication intermediates. Thus, SDE1/SGS2 and SDE3 would be redundant (Fig. 9.2). Also consistent with a role of SDE1 and SDE3 in dsRNA synthesis, it was shown that VIGS of the phytoene desaturase gene mediated by TRV is unaffected in both *sde1/sgs2* and *sde3* (Dalmay et al., 2000; Dalmay et al., 2001). This result clearly locates these two SDE factors upstream of the dsRNA processing and ssRNA degradation steps.

9.4.4.2 The spreading of target sites observed in sense transgene RNA silencing requires SDE1

Another line of evidence that (at least) SDE1 is required for dsRNA synthesis comes from the original observation, made in the GFP transgenic *N.benthamiana*, that the regions of the epiGFP RNA that are targeted in systemic silencing extend beyond the sequence of the triggering epiGFP construct (Chapter 5). It was proposed that this "spreading" of target sites occurred at the RNA level and involved the activity of an RdRp that would synthesise intGFP dsRNA using aberrant intGFP ssRNA as templates. This hypothesis has been recently tested in GFP transgenic *Arabidopsis* and it was

confirmed that spreading is compromised in the *sde1* mutant, while it occurs in wild type GFP plants (Fabian Vaistij, unpublished data).

9.4.4.3 Exploiting spreading

Shortly after the discovery of the "spreading" phenomenon (chapter 5), *N.benthamiana* plants were transformed with chimaeric transgene constructs in which the GFP ORF was interrupted with a fragment of an endogenous gene sequence (PDS, in this example Figure 9.3). One stable transformant was recovered, which contained an insert of the GF/PDS/P chimaeric construct. If spreading was not a peculiarity of the GFP sequence, it was predicted that, upon activation of silencing with GFP-specific sequences, (for example with a recombinant virus such as TRV-GF, Figure 9.3), spreading would occur on the fusion RNA. Thus, the resulting putative dsRNA would carry PDS sequences. As a result, new species of 21-23nt RNA with PDS sequence would be produced and would target the endogenous PDS mRNA for degradation. As shown in Figure 9.3-A, the outcome of the TRV-GF infection in this line was consistent with these predictions because the infected plants exhibited the characteristic photobleaching phenotype associated with silencing of PDS (chapter 3). Production of 21-23nt PDS RNA was subsequently confirmed in these infected plants (Fabian Vaistij, unpublished data).

This result has important practical implications because it indicates that "spreading" could be used as a high throughput silencing technology in transgenic *Arabidopsis* plants that exhibit PTGS of GFP. Such lines can be used as "recipients" for GFP-fusion constructs that would contain exon sequences of particular endogenous genes. These

constructs would be introduced in the GFP silenced plants by *Agrobacterium*-mediated seed transformation, which can be optimised so that a high proportion of primary transformants is generated (Figure 9.3-B). In the super-transformed plants, silencing initiated against GFP would spread on the fusion RNA, and the corresponding endogenous gene would be targeted. So far, I have tested this idea with two *Arabidopsis* endogenous genes. The preliminary results are encouraging, as a high proportion of primary transformants exhibited the expected silencing phenotypes (data not shown). It remains to be determined whether all of the transformants are actually silenced, and whether there is a variation in the strength of silencing between individual lines. More constructs are also currently being investigated, and they include sequences of genes required for lateral root formation (*KOJAK*) (Favery et al., 2001), for disease resistance (*LSD1*) (Dietrich et al., 1997) and for flower development (*SUPERMAN*) (Jacobsen and Meyerowitz, 1997).

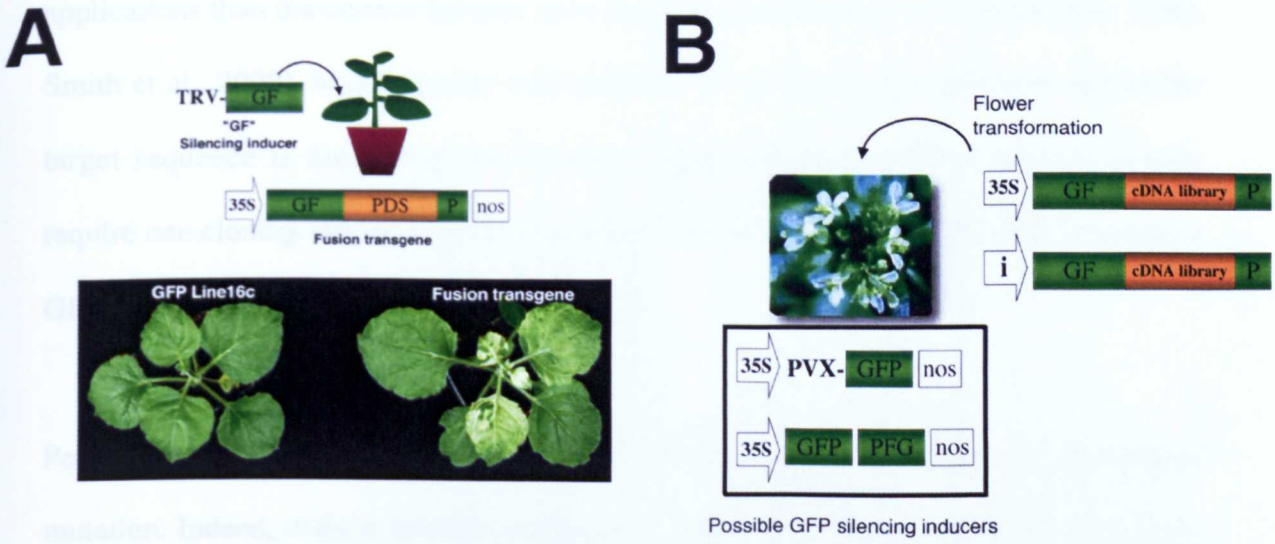

Figure 9.3. Exploiting "spreading". (**A**) Spreading of silencing affects the phytoene desaturase (PDS) endogneous gene in transgenic *N.benthamiana* carrying a GFP-PDS fusion construct. Spreading on the fusion RNA is initiated with a "GF" inducer of silencing provided here by a recombinant TRV vector that contains a GFP 5' DNA fragment. Infection of the transformant carrying the fusion transgene causes photobleaching, indicative of PDS silencing (right). Infection of a regular GFP transgenic line (16c, here) does not lead to photobleaching, confirming that the effect is specifically caused by the fusion construct. (**B**) Spreading as a high throughput silencing technology in *Arabidopsis*. Plants exhibiting PTGS of GFP are used as starting material. Consistent and heritable GFP silencing can be achieved by constitutive expression of a cDNA encoding the full-length PVX-GFP vector (technique currently used in the laboratory) or by expression of stem-loop RNAs (see section 9.4.1). GFP silencing plants are then super-transformed with a generic sense GFP construct allowing straightforward cloning of a normalised *Arabidopsis* cDNA library. Primary transformants are selected and should exhibit a silencing phenotype corresponding to the cDNA fragment carried by the fusion transgene. A refinement of this system is the use of inducible GFP-fusion constructs (promoter marked "i" on the scheme) allowing precise control of the silencing, both in space and time, as with a conditional mutant. Identification of the silenced gene would be by simple PCR using GFP oligonucleotides that are flanking the insert in the fusion transgene.

In theory, this system would be much more amenable to high throughput silencing applications than the current hairpin-based technologies (Chuang and Meyerowitz, 2000; Smith et al., 2000), which require manipulation of each individual construct so that the target sequence is duplicated and inverted. Spreading-based technology would only require one cloning step in which a normalised cDNA library would be mobilised into a GFP transgene construct.

Perhaps the best prospect for using spreading-based technologies is for conditional gene mutation. Indeed, if the super-transformed GFP-fusion locus is cloned under the control of an inducible promoter (Figure 9.3), it is predicted that silencing of the endogenous gene will only occur upon transcriptional activation of the fusion locus. This system is currently tested with a *UFO-GFP* fusion construct under the control of the ethanol-inducible promoter. UFO is required for differentiation of flower organs (Levin and Meyerowitz, 1995) and its silencing, upon ethanol treatment, should thus be easily scored

.

9.4.4.4 CMV: an exception?

An unresolved aspect of the model in Fig. 9.2 concerns the feature that makes some transgene ssRNAs specifically recognised as templates by SDE1/SGS2. As proposed in the previous chapters, it is possible that these RNAs have a distinctive structural feature or aberration. From studies in *A.immersus* (Barry et al., 1993), *Paramecium* (Ruiz et al., 1998) and more recently in *Chlamydomonas* (Wu-Scharf et al., 2000), it is possible that misprocessed or prematurely terminated RNAs are involved. As in maintenance of

transgene-induced systemic silencing (section 9.2.1) production of the aberrant RNA from transgene loci could be associated with DNA methylation or other changes at the chromatin level. The requirement for DNA or chromatin modification in sense transgene RNA silencing may be illustrated by mutation of *QDE3*. The QDE3 protein is similar to RecQ DNA helicases (Cogoni and Macino, 1999) and is necessary for initiation and maintenance of quelling in *Neurospora crassa*.

As discussed in section 9.2.2, it is possible that some viruses produce aberrant RNA in the course of an infection, even in the absence of homologous nuclear DNA. This could be the case for CMV, the only RNA virus (among more than six tested) that is affected by the *sde1/sgs2, sgs3* or *sde3* mutations in the host plant (Mourrain et al., 2000). The hypersusceptibility to CMV could be explained if replication of CMV leads to production of the aberrant RNAs that are recognised by SDE1/SGS2.

Why are other viruses not affected by the *sde1/sgs2* mutation? It is possible that they do not produce aberrant RNAs, in which case silencing would only be activated by dsRNA produced as a replication intermediate. Alternatively, it could be that the viruses do produce aberrant RNAs, but that these RNAs are not recognised by SDE1. Perhaps the six SDE1/SGS2 *Arabidopsis* homologues are involved in the processing of different forms of aberrant RNAs. These could include transcripts of RNA viruses other than CMV, as well as RNAs produced by DNA viruses (note that the accumulation of DNA viruses in the *sde/sgs* mutants has not been investigated so far). This proposal is supported by the recent identification of a salycilic acid-induced RdRp, distinct from

SDE1/SGS2, that restricts accumulation of tobacco mosaic virus and PVX in tobacco (Xie et al., 2001).

9.4.5 A model for RNA silencing as a response to virus infections: possible implications

The speculative model in Figure 9.2 predicts that if one of the several *Arabidopsis* CAF homologues is indeed the Dicer relative, the corresponding mutant should be highly susceptible to many types of viruses and should contain decreased levels of virus-induced 21–23nt RNAs. Such mutant could also be useful for the isolation of the elusive dsRNA product of the SDE1 RdRp evoked in this discussion. Indeed, depletion of the dsRNA processing machinery may increase the stability of this molecule. Based on this model, it is also expected that the introduction of the *ago1* mutation in *Arabidospsis* plants where silencing is triggered by hairpin RNAs could indicate whether AGO1 is located upstream or downstream of dsRNA synthesis.

It is striking that, in contrast to the *sde* and *sgs* mutants (genes that are possibly involved in synthesis of dsRNA), the available mutants of *CAF* and of several members of the *ARGONAUTE/ZWILLE/PINHEAD* family (genes possibly involved in dsRNA processing and ssRNA degradation) show severe developmental defects. Null-mutations in *CAF/SIN1* are embryo-lethal. Perhaps these growth defects indicate that the core mechanism of silencing plays a crucial role in endogenous gene regulation. These strong developmental defects would explain why mutation screens for RNA silencing components in plants led only to identification of surveillance/amplification functions of SDE1/SGS2, SDE3 and SGS3. It is also likely that these screens have been strongly

influenced by the involvement of sense transgenes. Genetic identification of factors specifically involved in virus-induced RNA silencing, if accessible, will require alternative screens based, for example, on loss of recovery. Alternatively, it may be possible to screen for loss of silencing triggered by hairpin RNA constructs, which, according to the model, should enter the silencing pathway in a SDE-independent manner as shown for most RNA viruses. Ideally, this screen should be performed in a *sde1* mutant background because it cannot be ruled out that some RNA produced by the inverted repeat locus would be recognised as templates by SDE1 (i.e. some nonfolded RNAs). Investigation of the viral suppressors could provide a third alternative to identify such factors.

9.5 Mechanism of silencing suppression

9.5.1 Current constraints in positioning the suppressors in the silencing pathway

At present, little is known about the targets of viral-encoded silencing suppressors. In addition, their modes of action have been largely inferred from overexpression experiments in different transgene-induced silencing systems. However, it is becoming clear that the experimental plant material used to study the suppressors, as well as the mode and timing of their expression has a significant influence on the outcome of such approaches. For instance, most factors have been identified through their capacity to suppress transgene-induced RNA silencing, which is triggered in different ways in plants. Thus, a suppressor targeted against an SDE-1-associated step might not be recognised if

it is studied in a transgenic system in which silencing is genetically determined by production of hairpin RNAs (Fig. 9.2).

A second constraint in the study of silencing suppressors might be the co-evolution between viral-encoded suppressors and hosts. This concept was evoked in section 9.3.2 to explain the frequent variations in the efficiency of suppressors isolated from phylogenetically related viruses. Co-evolution means that the inferences made on the mode of action of a particular suppressor can be influenced by the choice of the suppressor itself (strong or weak alleles from related viruses) and the choice of the host in which it is studied. It is also likely that expression of suppressors outside the context of their naturally encoding virus, either as highly transcribed transgenes or from heterologous viral vectors (as done in most studies, including the ones described in this thesis) provides an exaggerated picture of their actual involvement in normal infections.

Suppressors are often identified or studied in silencing reversal assays, a rapid method of investigation (Chapter 5,6). However, in normal infection, silencing would coincide with, rather than precede, suppression. The images in Figure 9.4 illustrate the dramatic influence exerted by the timing and mode of expression of the p19 protein on transgene-induced RNA silencing in *N. benthamiana*. Which phenotype is more informative about the mode of action of p19? One where silencing precedes suppression (panels a and b) or one where silencing occurs simultaneously to suppression (panels c and d)?

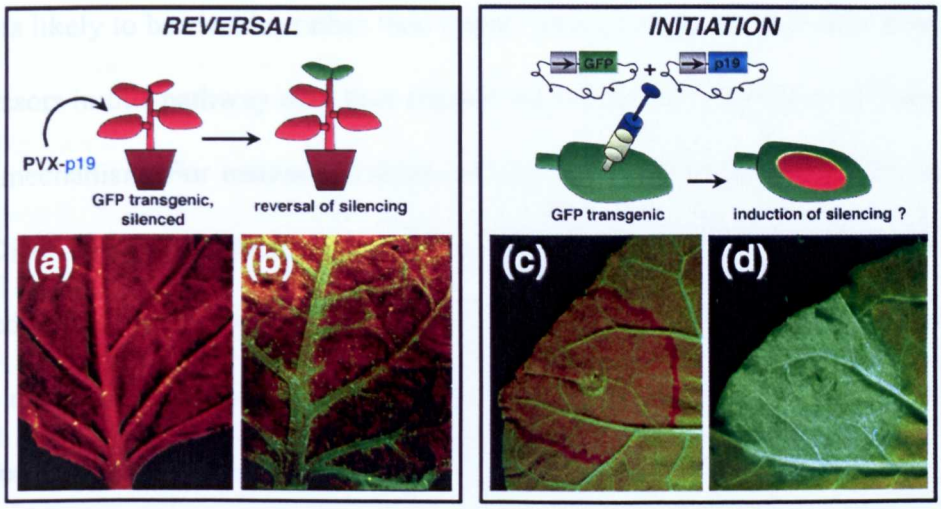

Figure 9.4. The timing and mode of expression of the tomato bushy stunt virus (TBSV) p19 protein dramatically influences the outcome of silencing suppression assays. In the reversal assay (left-hand panel), p19 is expressed from a recombinant PVX vector that is inoculated onto a silenced GFP transgenic plant. Figure a shows a leaf of a transgenic plant in which GFP is uniformly silenced, hence the red colour under UV illumination. Figure b shows the reversal of GFP silencing in this plant (limited to the vicinity of the veins) induced in new emerging leaves by infection of PVX expressing the p19 protein. The right-hand panel shows the initiation assay in which ectopic copies of a gene expressing GFP are locally introduced into the leaf of a nonsilenced, GFP-expressing plant. Ten days after treatment (c) the inoculated area appears red as a result of GFP silencing initiated by the ectopic, homologous DNA. If a similar experiment is performed in the presence of a gene expressing p19 (d), silencing is compromised, leading to extreme levels of ectopic GFP expression, as evidenced by the intense green fluorescence observed under UV.

Such contrasting phenotypes probably reflect the complexity of the silencing pathway, which is likely to be circular rather than linear, with multiple entry points. Positioning the suppressors in this pathway may thus require the biochemical isolation of individual steps in the mechanism. For instance, Tamas Dalmay has recently set up a biochemical assay for a Dicer-like activity in *N.benthamiana* and *Arabidopsis* (by measuring processing of labelled dsRNA into 21-23nt RNA products). Performing the assay with extracts of leaves containing silencing suppressors would indicate whether some of these factors compromise the dsRNA processing step of PTGS. In principle, viral-encoded suppressors could affect – directly or indirectly – any step of the model depicted in Fig. 9.2 One could argue, however, that factors targeted against silencing components that are essential to the plant (for example, AGO1) would be naturally selected against, unless suppression occurs as a regulated process.

9.5.2 Viral suppression of systemic silencing

9.5.2.1 Can we use the silencing suppressors to elucidate the nature of the systemic silencing signal?

In chapter 8, the effect of the PVX-encoded p25 protein on systemic and local silencing of a GFP sense transgene was analysed. Although p25 inhibited the intracellular silencing induced by a GFP sense transgene, it had no effect on the silencing triggered by replication of PVX-GFP. This result indicates that the p25 target is in the SDE/SGS part of the pathway, which is necessary for transgene-induced, but not for virus-induced silencing. In contrast, systemic spread of the silencing locally induced by PVX-GFP was inhibited by p25, suggesting that signalling, unlike to intracellular silencing, requires the SDE/SGS processing of the PVX-GFP RNA. A possible target of p25 is SDE3, which like p25 belongs to the class I superfamily of RNA helicases. Since these RNA helicases are known to function as homodimers, it is conceivable that p25 could bind and thereby inactivate SDE3. As SDE3 is likely required for the function of SDE1, this interaction would also affect indirectly dsRNA processing by SDE1. This hypothesis is highly speculative and remains to be experimentally tested.

As discussed in chapter 8, an indirect implication of our findings with p25 is that the systemic signal might not be the 21-23nt RNA but, instead, a dsRNA precursor of these molecules. Recent grafting experiments in transgenic tobacco expressing the TEV HcPro appear to be consistent with the suggestion that the systemic silencing signal is a nucleic acid distinct from the 21-23nt RNAs. In this experimental system, stable expression of

HcPro eliminates production of the 21-23nt RNA in transgenic lines producing a systemic signal targeted against the GUS RNA (Mallory et al., 2001). However, synthesis or movement of the silencing signal is not prevented by HcPro because non silenced GUS transgenic scions become silenced when grafted onto those plants (Mallory et al., 2001). However, a drawback in these experiments is that the suppression effect of HcPro is not the same throughout the plant growth, as indicated by MUG assays performed at different developmental stages. Therefore, it cannot be excluded that some 21-23nt RNAs were synthesised and transported into the scions before the full effect of HcPro was monitored. In addition, the failure to detect 21-23nt RNA in Northern blot analysis is not a strict indication that they are not produced at all. Thus, the precise nature of the systemic silencing signal remains an open question.

9.5.2.2 Two silencing signals?

It was shown in chapter 4 that a line of red fluorescent tissue appears consistently at the edge of epiGFP-infiltrated tissues of transgenic GFP *N.benthamiana* (Figure 4.2). Interestingly, this red line seemed to correspond to silenced tissues that were located outside the infiltrated area itself, since it could be readily detected at 5 days post-infiltration ahead of tissues in which epiGFP expression was evident as enhanced green fluorescence, as assessed under UV illumination. To allow precise identification of the cells that received the T-DNA and the cells exhibiting silencing, we used an *Agrobacterium* strain carrying a T-DNA with GUS and GFP reporter genes (35S-GUS-35S-GFP, Figure 9.5-A). Histochemical staining of GUS was used to identify cells in which T-DNA transfer had taken place (Figure 9.5-A, panels 1 and 3). Loss of GFP

fluorescence (Figure 9.4, panels 2 and 4) was the indicator of silencing. When the GUS and GFP images were superimposed it was clear that the silencing had spread for 1–1.5 mm beyond the cells in which T-DNA transfer had taken place (Figure 9.5-A, panel 5). We concluded from these additional experiments that this zone indicated local spread of GFP silencing.

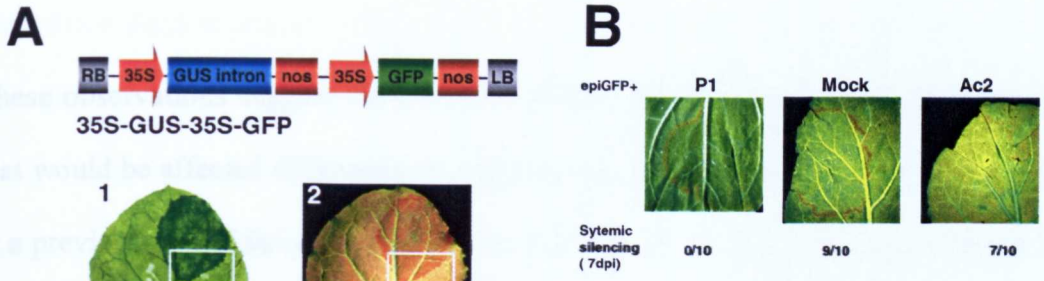

Figure 9..5. Two silencing signals? (**A**) Assay for cell-to-cell movement of the GFP silencing signal. The 35-GUS-35S-GFP construct was inserted into the T-DNA of the pBin19 binary vector. The GUS ORF contains an intron. The image in panels 1-4 are all of the same leaf and were taken at 12 days after infiltration of the 35-GUS-35S-GFP construct, when local PTGS of GFP was extensive in the infiltrated area. Panel 2 shows the infiltrated zone surrounded by a line of darker red fluorescent tissue that is evident in the close-up view in panel 4 (arrow). The leaf was subsequently stained for GUS activity (panels 1 and 3) and the staining was superimposed on the UV image of panel 4. Panel 5 indicates that PTGS of GFP clearly extends beyond the GUS-stained area (arrow) and coincides with the red line seen in panel 4. The bar represents 5mm. (**B**) Distinct effect of silencing suppressors on local and systemic silencing. The epiGFP construct was used to trigger silencing in GFP transgenic *N.benthamiana* line 16c in combination with (P1 and Ac2 panels) or without (Mock) expression of silencing suppressors. The appearance of the line of red fluorescent tissue and of systemic silencing was assessed at 7 dpi.

Following the mobilisation of the silencing suppressors in T-DNA expression cassettes, the effects of these viral proteins on local and systemic signalling was investigated using the transient expression assay described in chapter 8. Individual suppressor constructs

were used in *Agrobacterium* co-infiltration experiments, in combination with the epiGFP strain, as an inducer of local and systemic silencing in the GFP *N.benthamiana*. As shown in figure 9.5-B the RYMV P1 protein caused a loss of systemic silencing but did not prevent the appearance of the red line around the infiltrated patch (similar timing and extent as in the control experiment). Conversely, expression of Ac2 did not prevent systemic silencing but delayed by more than 7 days the appearance of local silencing.

These observations suggest the existence of two distinct silencing signalling molecules that would be affected differently by suppressors. This idea has already been put forward in a previous part of this discussion (section 9.2.1), to accommodate the apparent distinct requirements of the maintenance and local RNA degradation steps of systemic silencing.

9.5.3 Host-encoded suppressors of gene silencing

In contrast to p25, the potyviral HcPro apparently targets an intracellular step that is necessary for maintenance of silencing independently of whether it was initiated by a virus or a transgene (Anandalakshmi et al., 1998; Llave et al., 2000). A tobacco calmodulin-like protein, RgsCam, was shown to interact with the TEV HcPro in the yeast two-hybrid system. When virally or transgenically over-expressed, RgsCam mimics the suppression effects of the over-expressed HcPro itself (Anandalakshmi et al., 2000). In addition, expression of RgsCam, which is normally low in tobacco, is greatly enhanced by HcPro. These findings are intriguing, as they suggest that HcPro recruits a calcium-dependent regulatory pathway that negatively controls RNA silencing in plants. However, confirmation of this hypothesis awaits the characterisation of plants deficient

for RgsCam. These mutants should exhibit enhanced potyvirus resistance and, possibly, enhanced transgene-induced RNA silencing.

The finding of a potential plant-encoded silencing suppressor opens new perspectives, both experimentally and conceptually. First, it prompts new genetic screens for factors which, like RgsCam, would prevent RNA silencing when over-expressed. There are at least three ways to identify these factors. An activation-tagging strategy could be used, whereby the parental *sde/sgs* lines would be transformed with enhancer-trap constructs and subsequently screened for loss of RNA silencing. For example, this strategy has been used successfully in Pamela Green's lab to identify a factor that prevents the DST-mediated mRNA degradation in *Arabidopsis* (PSI symposium, Iowa, 2001). Alternatively, a *N.benthamiana* cDNA library could be engineered in a T-DNA over-expression cassette and used as suggested in the "forward approach" for identification of new viral-encoded silencing suppressors (section 9.35.2). In this case, enhanced green fluorescence caused in the *Agrobacterium*-infiltrated patch by over-expression of a host gene (a potential indicator of silencing suppression) would be scored under UV. A third alternative would be to look for increased levels of host mRNA in response to silencing suppressors. This approach should identify candidate host suppressors like RgsCam, whose RNA is up-regulated by HcPro. Changes in gene expression could be resolved by cDNA AFLP in transient expression systems (i.e. *Agrobacterium*-mediated transient expression of individual suppressors in *N.benthamiana*) or by micro-array analysis in stable *Arabidopsis* transformants. Most of these approaches are currently being investigated.

Conceptually, the idea of plant-encoded suppressors is consistent with a role for RNA silencing in plant development because it would allow both positive and negative control. The need for a down-regulation mechanism would be particularly important if the RISC-mediated RNA degradation is catalytic. There may be, however, several alternatives to endogenous suppressors for down-regulation of RNA silencing, as discussed in section 9.6.2.3.2.

Another intriguing result comes from an analysis of the CMV 2b protein. Although the protein is from a cytoplasmically replicating virus, silencing suppression requires nuclear localization of 2b (Lucy et al., 2000). This suggests that 2b could interfere with a nuclear step (or, possibly, some nuclear steps) of RNA silencing, or that the protein suppresses silencing by altering endogenous gene expression.

9.5.4 Identifying the targets of viral-encoded silencing suppressors

There are at least three possible ways to identify the targets of the various silencing suppressors. These are discussed below.

9.5.4.1 Physical interaction

As shown for RgsCam, it is possible to identify components of the silencing suppression mechanism by using the yeast two-hybrid system or related techniques. Alternatively, purification techniques based on immuno-precipitation can be envisaged. This would require the production of suppressor-specific antibodies, or the use of epitope-tagged suppressors. I have confirmed that several of the viral suppressors identified in this thesis retain their function when they carry a carboxy-terminal HA-tag. Therefore, a direct physical approach form *Agrobacterium*-infiltrated *N.benthamiana* tissues or *Arabidopsis* stable transformants is possible. A search for interactors with the HA-tagged P1 protein is underway in the laboratory (A. Herr).

9.5.4.2 Genetic approach

In the genetic approach, the *Arabidopsis sde/sgs* parental lines would be transformed with viral suppressors and plants exhibiting suppression of RNA silencing would be identified. These would then be mutagenised and subsequently screened for restoration of RNA silencing. There are at least two potential outcomes of this screen. One is the identification of plant-encoded suppressors of silencing which are recruited by the viral proteins, as suggested with RgsCam-HcPro (Anandalakshmi et al., 2000). The second possibility is the isolation of a host factor necessary for RNA silencing that would have

been modified by the mutation in such a way that it can still ensure its function but it can no longer interact with, or be inhibited by, the viral suppressor. This suggests that point mutation mutagens (EMS) rather than deletion mutagens (fast neutrons) should be used in this type of screen. It is also likely that this approach will require screening of a large number of mutagenised plants.

9.5.4.3 Changes in gene expression

Micro-array and cDNA AFLP-based technologies have been already proposed as potential ways to identify plant-encoded negative regulators of RNA silencing that are induced by some viral suppressors. In effect, these techniques would also allow detection of genes that are down-regulated by the viral suppressors. Down regulated host mRNA (at the transcriptional or post-transcriptional level) could correspond to possible targets of the silencing suppressors. For instance, the nuclear localisation of the 2b protein (Lucy et al., 2000) is compatible with it causing transcriptional repression of host gene(s).

9.5.4.4 VIGS

We have already discussed the various experimental evidence supporting the idea that RNA silencing is a branched pathway. One of the strong implications of this model is that some RNA silencing factors, for instance those required for dsRNA synthesis in sense transgene silencing, are actually dispensable for VIGS mediated by RNA viruses. This prompted the suggestion that VIGS could be potentially used to dissect some elements of the RNA silencing mechanism. This idea was recently tested in the laboratory with SDE3, which, according to the model, is not required for VIGS but should be required for

systemic silencing in *N.benthamiana* because it is an example of sense transgene silencing (Chapter 5). The cDNA for the *N.benthamiana* SDE3 homologue was cloned and used in VIGS experiments (using the TRV vector) that were performed on systemically silenced GFP plants (line 16c). It was shown that infection with TRV-SDE3, but not with the empty TRV vector, compromises systemic silencing in those plants (Louise Jones, unpublished data).

This result indicates that reverse and forward VIGS can be used as tools to dissect some parts of the silencing pathway and could therefore be equally exploited to identify putative targets of the silencing suppressors. This could be easily done, using the assay described in section 9.3.5.1, in which transient expression of a suppressor and epiGFP causes a strong enhancement of the green fluorescence observed in leaves of *N.benthamiana*. This test could be performed with HcPro, p19, p25 and 2b, suppressors for which the enhance fluorescence has been confirmed (data not shown). A forward VIGS approach based on the TRV vector (where several thousands plants will be inoculated with individual silencing constructs), has been scheduled in our group and this represents a good opportunity to test the idea. Once silenced, plants would be treated with each epiGFP-suppressor *Agrobacterium* mixes and scored a few days later for compromised enhanced green fluorescence, assessed under UV illumination. As a preliminary test for this approach, RgsCam will be silenced with the TRV vector and the expected loss of suppression activity by HcPro will be tested.

It should also be noted that RNAi has been successfully employed to decrease (although not eliminate) the Dicer mRNA levels in *Drosophila*. It was confirmed that, in cells with reduced Dicer activity, RNAi of a reported gene mRNA was impaired. In effect, the authors used RNAi to suppress a gene required for RNAi.

9.5.5 An antiviral mechanism in animals?

Although, in animals, it is clear that RNA silencing plays a role in genome defence against transposable elements, there is still no evidence for it having an antiviral function. However, tools are available to address this important question. A recombinant Sindbis RNA virus (SIN) has been modified as a vector for endogenous gene overexpression in insects (Lewis et al., 1999). Engineering this virus with exon fragments, rather than full-length cDNA, could help to elucidate whether VIGS operates in host organisms of SIN. Existing evidence for an antiviral role is provided by an RNA-mediated process that causes nucleotide-sequence-specific resistance to the Rift Valley fever RNA virus in mosquito cells (Billecocq et al., 2000). Additionally, in mammal cells, the replication shutoff of the flock house nodavirus (FHV) RNA1 is a host response distinct from the interferon response or apoptosis, which shares striking similarities with recovery in plants (Johnson and Ball, 1999). FHV replication is supported in insects, fungi and plants, organisms in which RNA silencing is extensively studied. This virus could thus provide a universal tool to investigate the antiviral function of RNA silencing and also, possibly, some aspects of silencing suppression. Indeed, the RNA1 shutoff is reminiscent of the effect of HcPro depletion in potyviral genomes.

9.6 Other possible roles of RNA silencing in plants

9.6.1 Viruses and transcriptional gene silencing: from antiviral response to genome defence

Cauliflower mosaic virus (CaMV) is a pararetrovirus with a DNA genome amplified in the nucleus and expressed from the 35S and 19S RNAs. In addition to RNA silencing, CaMV can induce methylation and transcriptional inactivation of integrated nuclear transgenes driven by the CaMV 35S promoter (Al-Kaff et al., 1998). Whether this phenomenon is RNA-directed or results from DNA–DNA interactions between viral episomes and transgene promoter sequences, or a combination of both, is unknown. However, this observation suggests that transcriptional gene silencing targeted against the 35S promoter could be naturally involved in defence against CaMV. There are also reports of integrated pararetroviral-like sequences in plant genomes (Jakowitsch et al., 1999), which could contribute to transcriptional repression of infectious episomal genomes, through homology-dependent interaction.

Sequence-specific *de novo* methylation of homologous transgene DNA is not an exclusive feature of DNA viruses. For instance, VIGS activated by recombinant PVX or TRV carrying transgene promoter sequences leads to promoter methylation and TGS of the corresponding transgene (Jones et al., 1999). These nuclear effects are difficult to explain in terms of antiviral defence because they are caused by cytoplasmic viruses with an RNA genome. Another puzzling aspect of this RNA-directed methylation concerns the

influence exerted by target DNA. Available data indicate that transgene constructs are strongly susceptible to this process, whereas endogenous sequences are not (Jones et al., 1999; Thomas et al., 2001). Perhaps the discrepancy reflects a role of RNA-directed methylation in protection of the plant genome against invasive DNA. In this view, the artificial effect of RNA viruses on transgenes could indicate that some steps of RNA silencing are part of a mechanism, acting at the DNA level, which prevents or reduces expression of foreign DNA elements through methylation. Retrotransposons, mobile DNA parasites with cytoplasmic RNA intermediates have been proposed as possible targets of this 'retrograde' defence system (Wolffe and Matzke, 1999): RNA silencing triggered in the cytoplasm by active retrotransposons could cause their coordinated transcriptional repression in the nucleus. One possible way to test this hypothesis would be to engineer RNA viruses with retroelement sequences and monitor the methylation status of the corresponding DNA in infected plants. Conversely, plant tissues in which mobilization of retrotransposons is activated (i.e. in protoplasts) could exhibit altered resistance against such recombinant viruses.

RNA-directed methylation is not only triggered by viruses but also by viroids (Wassenegger et al., 1994) – plant pathogens with a noncoding genome composed of circular RNA duplex – as well as transgene repeats producing double-stranded (ds)RNA (Mette et al., 2000). Collectively, these observations suggest that dsRNA, which is the form of replication of RNA viruses, could act as a trigger and/or mediator of RNA-directed methylation. Confirmation of this hypothesis would provide a link between

transcriptional and post-transcriptional silencing events in plants, because dsRNA also appears as a key molecule for activation of RNA silencing in the cytoplasm.

9.6.2 RNA silencing in plant physiology and plant development

Considerable attention is currently given to the idea that RNA silencing might be involved in the regulation of developmental/physiological processes. In plants, a number of indirect evidences support this proposal. For instance, the *sde1* and *sde3 Arabidopsis* mutants exhibit in common a number of aberrant features. Rosette leaves appear serrated and cauline leaves have a peculiar shape that makes them look like a "sickle" (Tamas Dalmay, unpublished observations). It is unlikely that this phenotype is due to secondary mutations carried by the mutants because (I) it is observed in several independent alleles of each mutation and (II) it is observed independently in both *sde1* and *sde3*, which, as discussed, are most likely defective in the same stage of RNA silencing. Interestingly, primary transformants of *Arabidopsis* plants that over-express some viral-encoded suppressors also exhibit a "sickle leaf" phenotype (data not shown). Another possible indication that RNA silencing might be involved in development is the severe abnormalities and the sterility of the *ago1* mutant. However, it is possible that this strong phenotype is due to pleitropic effects associated with the mutation, and not to the suppression of RNA silencing *per se*. Indeed, *sgs4* has been recently characterised as a hypomorphic allele of *ago1*. This mutant has little developmental defects but retain a full *suppressor of gene silencing* phenotype.

There are different ways to address directly the role of RNA silencing in plant development, as explained below.

9.6.2.1 Genome expression analysis

Genome expression analyses (cDNA-AFLP, micro-arrays) performed on the *sgs/sde* mutants or in plants expressing suppressors of RNA silencing may represent a valid approach. However, the choice of the plant tissues in which the analysis is performed will probably influence the outcome, as it is not obvious that the RNA silencing machinery would be equally recruited in all organs, or at all developmental stages. In the case of micro-array analyses, it would probably be necessary to use DNA-based chips, such as those produced by Affymetrix®, because mRNA that are down-regulated by gene silencing would likely be underrepresented on cDNA chips. This kind of analysis will probably require a significant amount of work to sort out which genes are actually directly down regulated by RNA silencing. For instance, a particular mRNA may be repressed because one of its positive regulators (i.e. a transcription factor) is itself targeted by RNA silencing.

9.6.2.2 Small RNAs

As a universal marker of RNA silencing-related processes, the 21-23nt could be used for the identification of endogenous silencing targets. Small RNAs isolated from various tissues could be ligated as concatemers, subsequently cloned and sequenced. One drawback of this approach is the risk of contamination (I) with 21-23nt RNA from retro/transposable elements that are putatively regulated by RNA silencing in plants and

(II) with small RNA species unrelated to silencing that accumulate as the result of other RNA turnover processes.

9.6.2.3 Rearranged and peculiar DNA loci

Since dsRNA is one of the possible molecules from which 21-23nt RNA are produced, it may be of interest to identify loci that possess a peculiar DNA organisation, allowing synthesis of transcripts that would adopt a dsRNA-like structure. In theory, it should be possible to use bioinformatic tools to detect such loci (genome-wide analysis in *Arabidopsis* for instance) There are several known examples of such loci in plants and I will briefly outline two of them.

9.6.2.3.1 The niv-571 allele

In *Antirrhinum*, most null alleles at the nivea (niv) locus are recessive to Niv+, and when homozygous, give white flowers rather than the red flowers of wild type plants (Bollmann et al., 1991). In contrast the niv-571 allele is semidominant. Although it gives white flowers when homozygous, pale flowers result when this allele is heterozygous with Niv+. Analysis of the niv-571 allele showed that it carries a transposable element Tam3 insertion and three copies of the niv gene, with one copy in inverse orientation. It was shown that the Tam3 insertion is not necessary for the niv-571 inhibition effect and that the allele acts in trans to inhibit expression of its Niv+ homologue. Moreover, the inhibition is reversible after meiosis. The niv-571 effect was initially proposed to result from direct DNA-DNA interactions between niv alleles, similar to that observed during transvection in *Drosophila*.

However, all the attributes of the niv-571 inhibition are strikingly reminiscent of RNA silencing and one possible way to reconcile the data is to propose that dsRNA-like transcripts are produced from the complex niv-571 locus. Thus, 21-23nt RNA synthesised from this molecule would cause the degradation, in *trans*, of the Niv+ mRNA, explaining the dominant effect of niv-571. A direct test of this hypothesis was to analyse the niv 21-23nt RNA content in Niv+/Niv+, Niv+/niv-571 and niv-571/niv571 plants. It was indeed found that these species accumulate specifically in the presence of the niv-571 allele and that their level correlates positively with the copy number of the niv-571 allele (Andrew Hamilton, unpublished data). Thus, the Niv-571 locus is probably an example of naturally occurring RNA silencing phenomenon involved in flower pigmentation

9.6.2.3.2 The AmRLK locus

The AmRLK (*Antirrhinum majus* receptor-like kinase) gene encodes a novel type of receptor kinase involved in the control of epidermal cell shape in petals. This gene possesses a 300 bp intron which contains three regulatory elements (32, 89 and 72 bp long, respectively) that are found in the promoter of *FAR*, a class B MADS-box gene involved in flower organ development. The promoter sequences in the AmRLK intron are organised in such a way that an antisense RNA coresponding to the 5' exon of AmRLK could be potentially produced from the locus, provided that the factors required for *FAR* transcriptional activation are available in the cells. In addition, *in situ* hybridisation has revealed that the accumulation of AmRLK and FAR mRNAs do not coincide in petals (Eric Lacombe, unpublished data). An attractive scenario would be that AmRLK is

actually negatively regulated through RNA silencing caused by annealing of sense and antisense strand of the 5' AmLRK exon. This down-regulation would be conditioned by the transcriptional activation of *FAR*, consistent with the *in situ* hybridisation data. This hypothesis is currently being tested (Eric Lacombe) as it predicts that 21-23nt RNA corresponding to the 5' exon of AmRLK should accumulate in tissues were *FAR* is active. If this model is true, AmRLK would represent the first example of a locus undergoing regulated RNA silencing in plants.

9.7 Conclusion

Significant advances have been made in our understanding of the role and mechanism of RNA silencing over recent years. There is now strong evidence that RNA silencing operates as an innate antiviral defence in higher plants. Consistent with this finding, many plant viruses have elaborated proteins that suppress various steps of RNA silencing, a necessary adaptation if they are to replicate and spread in a plant. Forward genetic screens in *Arabidopsis* have revealed distinct genetic requirements for the initiation of transgene-induced as opposed to virus-induced RNA silencing, suggesting that RNA silencing is a branched pathway that converges at the accumulation of dsRNA. Recently, fundamental biochemical analyses of RNAi in *Drosophila* have shed light on the core reactions of RNA silencing, whereby dsRNA is processed into 21–23nt RNA, which serves as guide for a sequence-specific nuclease. The development of powerful RNAi *in vitro* assays, the isolation and further characterisation of RNA silencing mutants, and the investigation of viral-encoded silencing suppressors provide an exciting and fascinating research ground for the coming years.

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Correction Note

The author, Olivier Voinnet has identified mistakes in panels 3.4; 5.7; 6.4/6.5 and 7.1 of this PhD thesis. The panels are identical to those found in figures of the original scientific publications upon which the PhD thesis is based. The mistakes in panels 3.4, 5.7 and 7.1 have been adequately addressed with the editors of the corresponding journals, and so the present addendum also refers to published editorial corrections or statements in relation to them. The errors in panels 6.4/6.5 have led to the retraction of the original study and so the key experiments were reproduced here in this corrigendum.

Olivier Voinnet recognizes these errors in the respective original publications as being his sole responsibility and apologizes for not having detected and corrected them during the writing phase, which was posterior to the publications of the corresponding papers.

Figure 3.4B, left panel (p. 63)

This panel is identical to the upper panel of Figure 5B found in Ruiz et al. (1998) originally published in *The Plant Cell*:

*Initiation and Maintenance of Virus-Induced Gene Silencing. (**1998**). Ruiz MT, Voinnet O and Baulcombe DC. Plant Cell, 10(6):937-46.*

The error concerns the 13 days postinoculation (DPI) data. These were incorrectly presented in a mock up image made during figure drafting, containing repeated copies of lanes 2,3,4, on the one hand, and of lanes 6 and 7, on the other. The corresponding original northern blots were retrieved, including independent biological duplicates of the faulty 13 DPI as well as replicates for the non-erroneous 20 DPI data sets. These were provided to the *Plant Cell* for full transparency. Upon independent peer review of the material and explanations provided, an amended figure and revised figure legend were issued in a correction notice published by the Plant Cell in July 2015 to which the reader is referred:

- *Correction to Ruiz et al. 10(6):937. (**2015**) Plant Cell, 27(7):2078-79.*

The corrected images show, as stated in the original article and in the corresponding section of the PhD thesis (p. 62-63), that PVX-GF levels are similar in the infected NT and GFP transgenic plants at 13 DPI, and only becomes significantly reduced due to the onset of gene silencing at 20 DPI time point and beyond in the GFP plants. *The Plant Cell* corrigendum points out that none of the original conclusions of the paper and, accordingly, of the PhD thesis, are affected by the correction.

Consequently, Figure 3.4 and its legend in the PhD thesis are hereby replaced by:

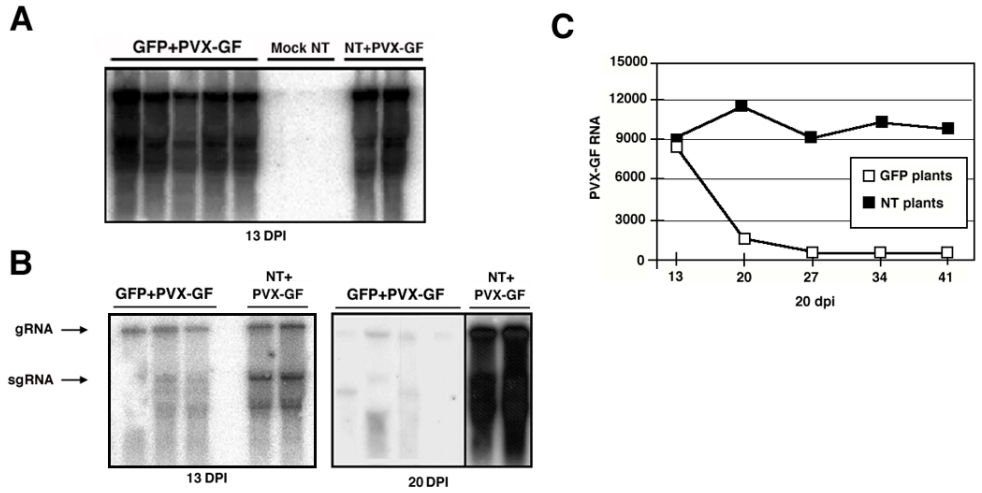


Figure 3.4. vGFP RNA levels. (**A**) vGFP in inoculated leaves. RNA samples were taken at 13 DPI from leaves of GFP8 (GFP) or non-transgenic (NT) lines that had been inoculated with PVX-GF. (**B**) vGFP RNA in systemically infected leaves. RNA samples were taken at 13 and 20 DPI from the uppermost systemic leaves of GFP8 (GFP) or non-transgenic (NT) lines inoculated with PVX-GF. In both (**A**) and (**B**), equal amounts (10 µg) of each RNA sample were fractionated by agarose gel electrophoresis and a phosphorus-32-labeled RNA probe for GFP was used to detect the recombinant viral RNAs. The genomic (gRNA) and major subgenomic (sgRNA) RNA species are labelled. Each sample was analysed in replicate and the gel on the right (20 DPI) was exposed longer than was the

gel on the left (13 DPI) to allow detection of the residual low levels of PVX-GF RNA in the samples from the GFP transgenic plants. **(C)** vGFP RNA in systemically infected leaves. RNA samples were taken at the indicated DPI from the uppermost systemic leaves of GFP8 (GFP) or non-transgenic (NT) lines inoculated with PVX-GF. Equal amounts (10 µg) of each RNA sample were fractionated by agarose gel electrophoresis and a phosphorus-32-labeled RNA probe for GFP was used to detect the recombinant viral RNAs. The level of vGFP gRNA in each sample was quantified in terms of PSL units using phosphorimaging equipment (see Methods). Each point represents the average value from three RNA samples.

Figure 5.7C (p. 110)

This panel is identical to Figure 6C found in Voinnet et al. (1998) originally published in *Cell*:

*Systemic Spread of Sequence-Specific Transgene RNA Degradation in plants Is Initiated by Localized introduction of Ectopic Promoterless DNA. (**1998**). Voinnet O, Vain, P, Angell S, and Baulcombe DC, Cell, 95: 177-187.*

The error concerns lanes 6 and 7 of the central panel in Figure 6C, of which the former is a duplication of the latter. Although the original blots used to mount panel 6C are no longer available, the duplication possibly occurred at the final mounting stage when the overall complexity of panel 6C probably prompted the rearrangement of lanes to facilitate its labelling The separations between the sections encompassing lanes 1-5 (A) , 6-10 (B) and 11-14 (C), respectively, suggest that they were assembled from excerpts of a larger membrane hybridized with an anti-GFP probe. The NT/non-bombarded(-) control (lane 6) of section B was likely located on the right edge of panel C, possibly just before lane 11 (panel C) given the samples' order in the experimentally equivalent Figure 6D. The strong similarities between all viral hybridization signals possibly caused the erroneous cut of lane 7 of section B instead of the cognate control lane from section C. The cut section was then likely positioned onto the original lane 6 of section B instead of the cognate bombarded(-) control lane that should have been taken from section C.

Without access to the original data, a correction of this figure is not possible. However, lane 6 is dispensable for data interpretation and an equivalent control lane is indeed absent in section C (lane 11-14) on the left. Moreover, this lane is not discussed in the PhD thesis. Finally, Figure 5.7D of the thesis and Figure 6D of the original publication depict similar results indicating that systemic silencing elicited by bombarded DNA derived from only a portion of the *GFP* mRNA produces silencing targeted against the non-overlapping portion, as established by nucleotide sequence-specific resistance displayed against recombinant PVX. Upon notification of the problem with Figure 6C of Voinnet et al. (1998), the following Editorial Note was issued by *Cell* in July 2016, to which the reader is referred:

● *Editorial Note. (**2016**) Cell, 166(3):779.*

"We the editors of Cell were contacted by the corresponding author, Dr. David Baulcombe, and the first author, Dr. Olivier Voinnet. They informed us that, in Figure 6C, lanes 6 and 7 were intended to show two different negative controls, but one of the lanes was erroneously duplicated. The authors were not able to locate the original data and could not determine how the error arose. Without access to the original data, a correction of this figure panel is not possible. Our evaluation of the other figures of the paper did not reveal any additional irregularity".

Consequently, lane 6 of Figure 5.7C is withdrawn from the PhD thesis. Figure 5.7C and its legend in the PhD thesis is hereby replaced by:

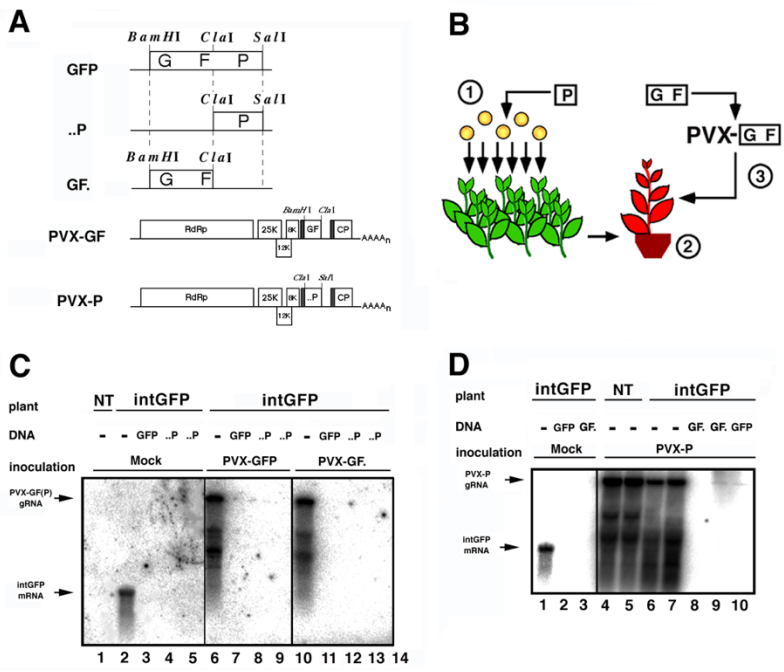


Figure 5.7. The regions of intGFP RNA that are targeted in systemic tissues expand beyond the sequence of the triggering epiGFP construct. (**A**) Bombarded epiGFP and inoculated viral constructs. The ..P and GF. DNA constructs are derivatives of the GFP construct described in Figure 4A. PVX-GF and PVX-P are PVX vectors carrying the GF. and ..P restriction fragments of the GFP open reading frame, respectively. Expression of the corresponding insert is controlled by a duplicated coat protein (CP) promoter indicated by shaded boxes (RdRp: RNA dependent RNA polymerase, 25K, 12K, 8K: cell-to-cell movement proteins, CP: coat protein). (**B**). Diagram illustrating the order of events described below. (**C**) Northern analysis of intGFP and PVX-GF/GFP RNAs. First, intGFP seedlings were bombarded with either uncoated gold particles (-) or gold particles coated with either the GFP or the ..P construct (see panel B①). After 21 days, when intGFP was systemically silenced (see panel B②), two upper leaves were inoculated with either water (Mock), PVX-GFP or PVX-GF (see panel B③). Five days after virus inoculation, total RNA was extracted from one of the two inoculated upper leaves and Northern analysis of 10 µg of RNA was carried out to detect accumulation of the intGFP and PVX-GF/GFP RNA (indicated on the left side of the upper panel). (**D**) Northern analysis of intGFP and PVX-P RNAs. The analysis was performed as described in (C), following inoculation of PVX-P to GF-bombarded intGFP plants.

Note that the phenomenon discovered in the experiments depicted in Figure 5.7, called “transitivity”, was subsequently characterized genetically in plants and linked to the extent of systemic silencing and spread of DNA methylation on the affected transgene via the synthesis of so called “secondary siRNAs”. Transitivity has now been demonstrated in virtually all RNAi-prone model organisms (e.g. fungi, *C. elegans*) encoding a specific class of enzymes called RNA-dependent RNA polymerases (RdRp) and was reported in many independent publications, among which:

Vermeersch L, *et al.* (2014). Detection and investigation of transitive gene silencing in plants. Methods Mol Biol.1112:219-41. ● Sijen *et al.* (2007). Secondary siRNAs result from unprimed RNA synthesis and form a distinct class. Science. 315:244-7. ● Bleys A, *et al.* (2006) Down-regulation of endogenes mediated by a transitive silencing signal. RNA. 12:1633-9. ● Petersen BO, Albrechtsen M. (2005) Evidence implying only unprimed RdRP activity during transitive gene silencing in plants. Plant Mol Biol. 58:575-83. ● Vaistij FE, Jones L, Baulcombe DC. (2002). Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. Plant Cell. 14(4):857-67.

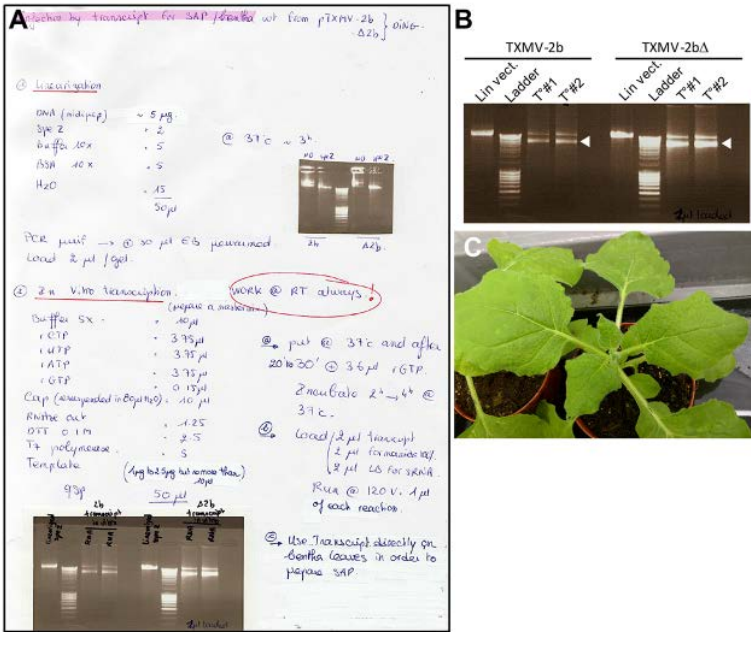
Figure 6.4/6.5 (p. 133-134)

The two panels are identical to Figure 5B and 6I found in Brigneti et al. (1998) originally published in *the EMBO Journal* :

*Viral pathogenicity determinants are suppressors of transgene silencing in Nicotiana benthamiana. **(1998)**. Brigneti G, Voinnet O, LI W-X, Li L-H, Ding S-W and Baulcombe DC. EMBO J. 17(22):6739-46.*

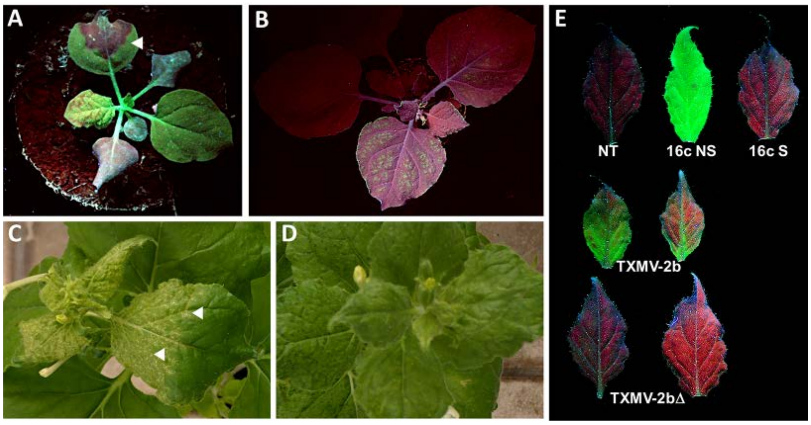
The irregularity concerns pixel duplications of background signal affecting the GFP signal panel in lane 1 of Figure 6.4 as well as lanes 7 and 9 of Figure 6.5. Although neither noted in the PhD thesis nor in the original publication, the two figures correspond to sections of the same RNA blot hybridized with the same radioactive probe. The aesthetical alterations were most likely made to remove dust marks or blemishes from the corresponding sections of the gel, which is no longer available. After being alerted of the irregularity, and given the unavailability of the originals, EMBO J stated that the most appropriate measure was to issue a retraction statement, published in August 2015 on the ground that the affected sections of the gel may have possibly contained experimental GFP signals, in particular in lane 9 purporting to show the absence of silencing reversion by a mutant allele of the 2b protein. Although an independent investigation commission recommended the reproduction of the experiment for a correction to be made, the decision of EMBO J on the matter is sovereign and was respected.

To correct these errors, the experiments were independently reproduced at O. Voinnet's requet in the spring 2015 by Mme Shahinez Garcia (SG) who conducted the molecular biology and Mr Christophe Himber (CH) who conducted the *in planta* systelic GFP silencing induction and the infections. SG and CH were two senior engineers of the former Voinnet group at the *Institut de Biologie Moléculaire des Plantes* (IBMP-CNRS, Strasbourg, France). The images presented in Additional Figure 1 are excerpts of SG's laboratory book and the plant pictures under normal and UV light presented in additional Figures 1 and 2 were taken by CH.



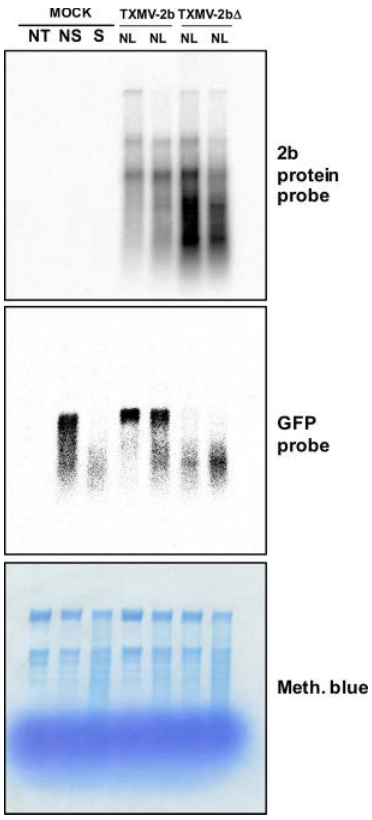
Additional Figure 1. (**A**) Snapshot of SG's laboratory book detailing the linearization and in vitro transcription of the TXMV-2b/2bΔ vectors. (**B**) In vitro transcripts (arrows) obtained for each recombinant virus in two independent reactions (T°#1, #2); Lin vect: linearized vector. (**C**) typical early mosaic symptoms caused by infectious TXMV-2bΔ transcripts ~15DPI of wild-type *N.benthamiana* plants.

Based on the shared natured of the two Figures, the GFP signals in lanes 1-3 serve as positive and negative controls for both the CMV (Thesis Figure 6.4, publication Figure 5B) and TXMV-2b/2bΔ (Thesis Figure 6.5, publication Figure 6I) infections. Because, furthermore, lanes 4-6 in Figure 6.4 are not contentious, it was thus decided that the experiments presented in Figure 6.5 would be independently reproduced. To that aim, the original TXMV-2b/2bΔ clones were obtained from Pr. Shou-Wei Ding (University of California, Riverside, USA), co-author on the original EMBO J 1998 publication. The two plasmids where received directly, in spring 2015, by SG. Briefly, and as originally described, 5µg of amplified DNA was linearized with the Spe-I restriction enzyme and used as a template for *in vitro* transcription by T7 RNA polymerase in a total volume of 50µl and in presence of 5'(m7Gp) (Additional Figure 1A). 2µl of each transcription reaction were then loaded onto a 100% formamide gel to verify the yield and integrity of viral transcripts (Additional Figure 1B). Transcription mixes were then directly rub-inoculated with Celite (SiO_2) onto leaves of 15-20 days old non-transgenic *N. benthamiana* plants. Immediately upon appearance of typical PVX chlorotic symptoms (Additional Figure 1C), sap extracts of TXMV-2b- an TXMV-2bΔ-infected plants was prepared in 5mM Na2HPO4 buffer and aliquoted in 100µl vials stored at -20°C. In parallel, CH set up systemic silencing in line GFP 16c (Add-Figure 2A-B) as reported originally (Brigneti et al. 1998). Briefly, leaves of 3 weeks old seedlings were infiltrated with the epi-GFP strain of *Agrobacterium tumefaciens*, as described Chapter 5, section 5.3.1 of the PhD thesis. 15-20 days later, systemically silenced GFP plants were inoculated with TXMV-2b- or TXMV-2bΔ sap extracts, with the former causing, as reported (Brigneti et al. 1998), significantly stronger symptoms than the latter (Add-Figure 2C-D) owing to enhanced virulence caused by functional 2b protein expression. Also as reported (Brignetti et al., 1998) and confirmed later independently (Li et al. 1999; Lucy et al. 2000) reversion of GFP accumulation monitored throughout time under UV illumination was detected only in young emerging leaves of TXMV-2b-, but not TXMV-2bΔ-infected plants at ~21 DPI onward (Additional Figure 2E; data not shown). Independent samples of young leaves were thus collected and stored at -80°C in the summer 2015; they were not analysed



Additional Figure 2. (A) Systemic silencing induction a few days post-infiltration of the epiGFP *A. tumefasciens* strain. The arrow indicates the infiltrated patch, which has truned completely red (GFP silenced) under UV light (**B**) Complete systemic silencing exhibited by plants used for the viral silencing reversion assay. The faint green fluorescent signal in one of the leaves is due to residual GFP protein accumulation at a time point when the GFP mRNA is below detection levels (**C-D**) TXMV-2b induces unusually strong symptoms including vein-centred flecks of necrosis (C; arrows) compared to the mild mosaic elicited by TXMV-2bΔ (D). (**E**) TXMV-2b but not TXMV-2bΔ causes GFP silencing reversion in new emerging leaves of silenced 16c plants (16c S). 16c NS: non-infected non-silenced 16c plants; NT: non-infected non-transgenic plants.

further due to the decision of EMBO J to retract the study. In May 2017, samples were repatriated from the IBMP to the ETH-Zürich to be molecularly analysed by biology engineer Gregory Schott, in the Voinnet group. Briefly, gel blot analysis of high molecular weight RNA was performed on 2µg total RNA extracted with the RNeasy Plant mini kit (Qiagen). RNA was separated on 0.9% (w/v) agarose formaldehyde gels for 2 hours at 110V in 1x HEPES pH7.4. Overnight RNA transfer was done by capillarity on a Hybond-NX membrane in 20x SSC, upon which total RNA was stained by Methylene blue after transfer and UV-crosslinking. Radiolabelled probes for detection of the GFP mRNA and multiple TXMV-2b/2bΔ RNA species were produced by random priming reactions in the presence of alpha-32P-dCTP. The template used were 700-bp-long (GFP) and 300-bp-long (CMV-2b) PCR products amplified respectively from mGFP5 and plasmid TXMV-2b (Forward primer: atggatgtgttgacagtagtg; reverse primer: tcaaaacgacccttcggcc). The membrane was first hybridized for GFP, stripped and re-probed for CMV-2b. In each case, five hours of hybridization in PerfectHyb buffer at 65°C were followed by 4 washes with 2x SSC, 0,1% SDS. Membranes were exposed for 12 hours (GFP probe) and 4 hours (CMV-2b probe) on a Phosphorimager screen. The results, shown in Add-Figure 3, confirm that no GFP signal above background is detected in mock-infected non-transgenic plants (original contentious lane 1, Figure 6.4 and 6.5) as is also the case in independent leaves of TXMV-2bΔ-infected silenced plants (original contentious lanes 7 and 9, Figure 6.5).



Additional Figure 3. See text for details. NT: non-transgenic, NS: non-silenced; S: silenced; NL: new leaf; Meth. blue: methylene blue staining.

Based on these results, lanes 1-3 of figure 6.4, dispensable for data interpretation, are hereby withdrawn from the PhD thesis. Consequently, Figure 6.4 and its legend in the PhD thesis become:

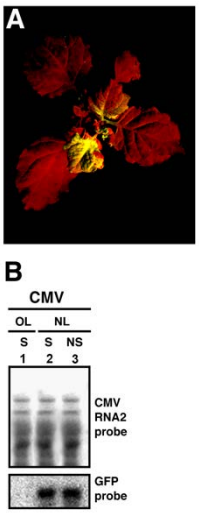


Figure 6.4. Suppression of PTGS by CMV. (**A**) GFP-silenced *N. benthamiana* (line 16c) infected with CMV (21d post inoculation). GFP expression was restored in the newly emerging tissue after systemic CMV infection had been established. (**B**) Northern analysis of RNA extracted at 15d post CMV inoculation from silenced (S) *N. benthamiana* plants of line 16c The RNA samples were taken either from old leaves (OL) that had emerged before systemic CMV infection or from new leaves (NL) emerging after CMV had spread systemically. 5 µg of total RNA per sample were fractionated by electrophoresis on a 0.9% (w/v) agarose-formaldehyde gel, blotted onto a nylon membrane and hybridised with probes specific for RNA2 of CMV or for GFP RNA, as indicated. The multiple RNA species hybridising to the CMV probe may represent degraded or subgenomic RNAs and have been described previously.

Additionally, Figure 6.5 and its legend in the PhD thesis are hereby replaced by:

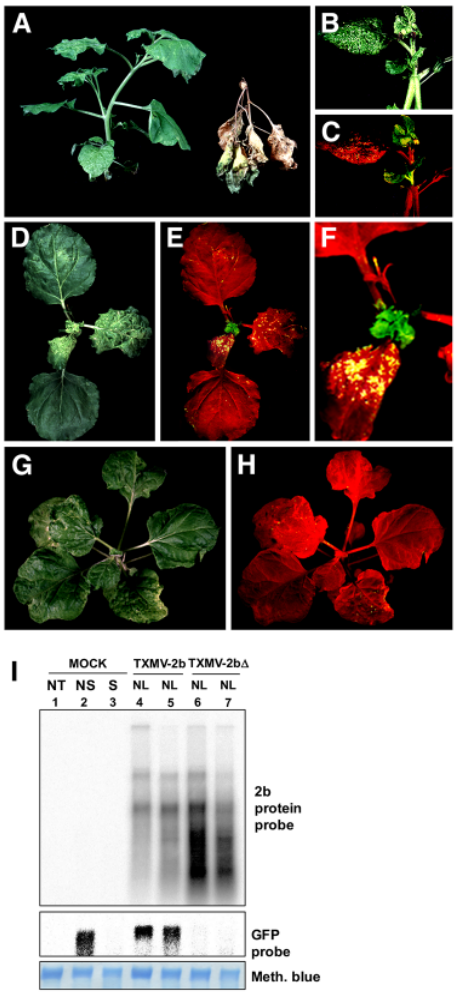


Figure 6.5. Suppression of PTGS by the 2b protein of CMV (**A**) Non-transformed *N. benthamiana* plants inoculated with TXMV-2bΔ (left) and TXMV-2b (right). The photograph was taken at 21 days post inoculation and both plants were the same age when inoculated. (**B**) GFP-silenced *N. benthamiana* line 16c showing symptoms of TXMV-2b infection at 21d post inoculation. (**C**) The same plant shown under UV illumination revealing the PTGS of GFP persisted in symptomatic leaves that had emerged from meristems before systemic infection but that it is suppressed in the post emergence leaves. (**D**) and (**E**) are aerial views of the plant shown in (B) under white light and UV illumination. (**F**) shows the apical zone from the image in (E). (**G**, **H**) GFP-silenced *N. benthamiana* line 16c showing symptoms of TXMV-2bΔ under white light (G) and UV illumination (H). (**I**) Northern analysis of RNA extracted 21DPI from either NT, non-silenced (NS) or silenced (S) plants inoculated with Mock, or from new emerging leaves (NL) of TXMV-2b- or TXMV-2bΔ-infected silenced plants. The RNA in NL was taken from samples from independent infection experiments. 5 µg of total RNA per sample were fractionated by electrophoresis on a 0.9% (w/v) agarose-formaldehyde gel, blotted onto a nylon membrane and successively hybridized with probes specific for the GFP mRNA and for the CMV-RNA2-encoded 2b protein as indicated. The various signals for the PVX genomic and subgenomic RNAs have been described already.

Note that identical results to those originally disclosed in Figure 5B and 6I of Brigneti et al. (1998) were obtained by Li et al. (1999) and Lucy et al. (2000) using the same systemic silencing induction method in line 16c and the same TXMV-2b/ 2bΔ vector as originally employed. The references are:

● *Strong host resistance targeted against a viral suppressor of the plant gene silencing defence mechanism. (**1999**). Li HW, Lucy AP, Guo HS, Li WX, Ji LH, Wong SM, Ding SW. EMBO J. 18(10):2683-91.*

● *Suppression of post-transcriptional gene silencing by a plant viral protein localized in the nucleus. (**2000**). Lucy AP, Guo HS, Li WX, Ding SW. EMBO J. 19(7):1672-80.*

Silencing suppression by 2b was demonstrated independently in multiple publications including:

Dong L, et al. (**2016**). Two amino acids near the N-terminus of Cucumber mosaic virus 2b play critical roles in the suppression of RNA silencing and viral infectivity. *Mol Plant Pathol*. 17(2):173-83. ● Du Z, et al. (**2014**). Nuclear-cytoplasmic partitioning of cucumber mosaic virus protein 2b determines the balance between its roles as a virulence determinant and an RNA-silencing suppressor. *J Virol*. 88(10):5228-41. ● Duan CG, et al. (**2012**) Suppression of Arabidopsis ARGONAUTE1-mediated slicing, transgene-induced RNA silencing, and DNA methylation by distinct domains of the Cucumber mosaic virus 2b protein. *Plant Cell* 24(1):259-74. ● Zhang X, et al. (**2006**). Cucumber mosaic virus-encoded 2b suppressor inhibits Arabidopsis Argonaute1 cleavage activity to counter plant defense. *Genes Dev* 20(23):3255-68.

Figure 7.1 (p. 147)

This panel is identical to Figure 1D found in Voinnet et al. (1999) originally published in *PNAS:*

*Suppression of gene silencing: A general strategy used by diverse DNA and RNA viruses of plants. (**1999**). Voinnet O Pinto Y and Baulcombe DC. PNAS, 96(24):14147-*

The error concerns the ribosomal RNA track of panel 7.1D purporting to show equal loading of the RNA samples, which was duplicated at the stage of figure mounting from the equivalent panel of Figure 7.2D displaying an identical 9-track format. After being contacted and consultation of expert editorial board members, *PNAS* issued a corrigendum in August 2015, to which the reader is referred:

●*Correction. (**2015**) PNAS, 112(34). doi:10.1073/pnas.1512971112.*

The correction notice, issued by the authors, states that the loading control duplication made by author Olivier Voinnet does not compromise the interpretation of the results. Indeed, as was established upon inspection of raw data of these period, total RNA was systematically quantified by spectrometry (260/280nm absorbance) followed by preparatory agarose gel electrophoresis to ensure equal loading of the smaple. Silencing suppression by the geminiviral AC2 protein (also referred to as C2, L2, AL2 or TrAP depending on the geminivirus under consideration) was later confirmed in multiple independent publications, including:

Kumar V, et al. (**2015**). Mungbean yellow mosaic Indian virus encoded AC2 protein suppresses RNA silencing by inhibiting Arabidopsis RDR6 and AGO1 activities. *Virology*. 486:158-72. ● Soitamo AJ, et al. (**2012**) Expression of geminiviral AC2 RNA silencing suppressor changes sugar and jasmonate responsive gene expression in transgenic tobacco plants. *BMC Plant Biol*. 12:204. ●Yang X, et al. (**2007**). Functional modulation of the geminivirus AL2 transcription factor and silencing suppressor by self-interaction. *J Virol*. 81(21):11972-81. ● Dong X, et al. (**2003**). Functional characterization of the nuclear localization signal for a suppressor of posttranscriptional gene silencing. J Virol. 77(12):7026-33. ● Van Wezel R et al. (**2002**). Mutation of three cysteine residues in Tomato yellow leaf curl virus-China C2 protein causes dysfunction in pathogenesis and posttranscriptional gene-silencing suppression. *Mol Plant Microbe Interact.* 15(3):203-8.

Consequently, the rRNA track of Figure 7.1 is hereby withdrawn from the PhD thesis. Figure 7.1 and its legend in the PhD thesis is hereby replaced by:

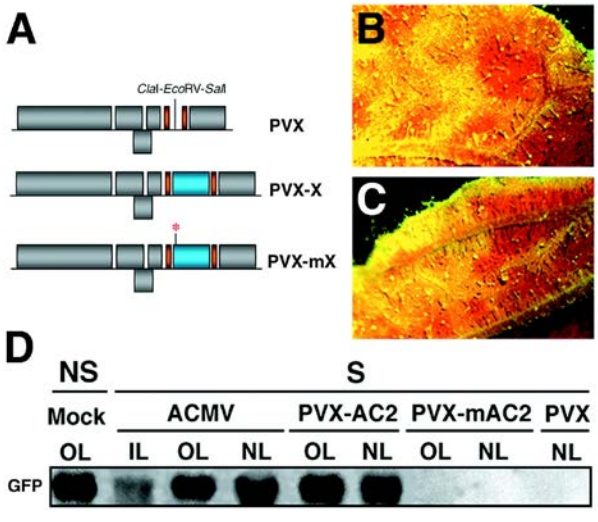


Fig. 7.1. Suppression of PTGS by ACMV and PVX-AC2. (**A**) Schematic representation of the PVX vector used to express various pathogenicity determinants tested in this study (referred to as "X"). Individual sequences were inserted into the P2C2S PVX vector using the ClaI–EcoRV–SalI multiple cloning site (19), leading to "PVX-X". Expression of the inserts (X, depicted as a blue box) and the PVX coat protein is controlled by duplicated coat protein promoters (indicated by a solid orange bar). Mutant versions of all pathogenicity determinants, referred to as "mX," were also used in this study (mutation indicated by a red asterisk). (**B**) Close-up image of an ACMV-infected leaf from a GFP-silenced N.

benthamiana. (**C**) Close-up image of a PVX-AC2-infected leaf from a GFP-silenced N. benthamiana. Photos from B and C were taken under UV illumination from a dissecting microscope at 15 DPI. The red tissue corresponds to chlorophyll fluorescence under UV and, thus, is indicative of gene silencing of GFP. The green fluorescent tissue that sometimes appears yellow is from expression of GFP and, thus, indicates suppression of gene silencing. (**D**) Northern blot analysis of RNA extracted at 20 DPI from either mock-infected, nonsilenced (NS), or silenced (S) N. benthamiana infected with ACMV, PVX-AC2, PVX-mAC2, or PVX. RNA samples were taken either from inoculated leaves (IL), old leaves that had emerged before the virus had spread systemically (OL), or from new leaves emerging after virus infection (NL). Equal amounts of each RNA sample (10 μg) were assayed by RNA gel blotting by using a 32P-labeled GFP cDNA as probe.
