REVERSAL OF RNA-MEDIATED GENE SILENCING PATHWAYS BY GEMINIVIRUS AL2 AND L2 PROTEINS

DISSERTATION

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RNA-directed gene silencing is a primary adaptive response by which plants defend against virus invasion. RNA-silencing pathways are complex, but can be divided into two basic categories: Post-Transcriptional Gene Silencing (PTGS) and Transcriptional Gene Silencing (TGS). PTGS occurs in the cytoplasm and is mediated by small interfering RNAs (siRNAs) 21-22 nucleotides (nt) in length generated from larger double-stranded RNAs by Dicer-like ribonucleases. One strand of the siRNA is incorporated into another ribonuclease-containing complex known as RISC (RNA-induced silencing complexes), where it targets homologous mRNAs for degradation. In addition to silencing at the site of infection, there is a mobile silencing signal of unknown composition that circulates throughout the plant, is amplified, and triggers systemic silencing. The second pathway, TGS, takes place in the nucleus. In this case 24 nt siRNAs program RISC-like complexes to methylation of cytosine residues in homologous promoter and coding regions of DNA, which encourages the formation of transcriptionally inactive heterochromatin by histone modification. TGS has primarily been thought of as a way to suppress the activity of potentially damaging native sequences such as transposons, but recent evidence has shown that it also can play an important role in defense against DNA viruses.
The AL2 and L2 proteins are related proteins encoded by geminiviruses of the *Begomovirus* and *Curtovirus* genera, respectively. AL2 is transcriptionally active, is required for the expression of late viral genes, and can also activate unknown host genes, whereas L2 is transcriptionally inactive. AL2 can suppress PTGS by two mechanisms. The first requires activation of host gene transcription by AL2 (transcription-dependent mechanism). The second mechanism is shared by AL2 and L2, and involves interaction with an inactivation of adenosine kinase (ADK), which is required for efficient cellular transmethylation activity. It has been previously shown that both AL2 and L2 can suppress silencing in an assay that likely measures the initiation PTGS. This suggests that AL2 and L2 can suppress PTGS initiation via a transcription-independent mechanism. In this thesis AL2, but not L2, is shown to also block systemic spread of PTGS by a transcription-dependent mechanism. In addition, AL2 and L2 are shown to reverse established systemic PTGS, which suggests interference with a maintenance step. However, while both AL2 and L2 reversed silencing in immature plants, silencing could only be reversed by AL2 in plants that had undergone the vegetative to floral transition. This suggests the existence of multiple, developmentally regulated silencing maintenance pathways. Finally, the role of methylation as a host defense was confirmed by studies which demonstrated that both AL2 and L2 can reverse TGS of a transgene and of native pseudogenes, and cause significant reversal of methylation throughout a plant genome. In this case, TGS reversal of most genes examined could be accomplished by the transcription-independent mechanism, although some require the transcription-dependent mechanism.
Taken together, the studies in this thesis further our understanding of viral pathogenesis and the nature of innate host defenses by demonstrating that AL2 and L2 can suppress both TGS and PTGS by multiple mechanisms.
Dedicated to my mother and my Flock
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CHAPTER 1

INTRODUCTION

1.1 Geminiviruses

Members of the family *Geminiviridae* are single-stranded DNA (ssDNA) viruses that infect a wide variety of both monocot and dicot plant species, and are named for the distinctive dumbbell shape of their twin icosahedral capsids (Bisaro, 2006). Geminiviruses have long been important plant pathogens; they have recently been shown to have been the culprit in the earliest recorded plant disease in Japan over 1200 years ago (Saunders et al., 2003). In modern times, they are a major cause of crop diseases in tropical and subtropical countries, causing infection and severely affecting crop yields in plants ranging from grain legumes to cassava (Mansoor et al., 2003; Vanderschuren et al., 2007). They are classified into four genera, the *Begomovirus, Curtovirus, Mastrevirus* and *Topocuvirus*, depending on factors such as their insect vector, genome organization, and host range (Fauquet et al., 2003).
1.1.1 Geminivirus genome organization

Each geminivirus particle contains a circular single-stranded DNA ~2.5 to 3.0 kb in length. Except for nanoviruses, they are unique among plant viruses in having ssDNA genomes. Geminivirus genomes may be monopartite or bipartite. Monopartite geminiviruses, such as those of the genus Mastrevirus, Topocuvirus, Curtovirus, and Old World members of Begomovirus, have a single genome encoding all viral proteins. Bipartite viruses, including some members of the genus Begomovirus, have two separate genome components, designated A and B, each packaged in separate viral particles. The A component contains all information necessary for replication and encapsidation, while the B component provides functions needed for virus spread between cells and throughout the plant (Rogers et al., 1986; Sunter et al., 1987). Currently, there are two systems for naming geminiviral genes which are transcribed from double-stranded DNA (dsDNA) intermediates which also serve as replication templates (see below). In both systems, both genes and proteins are designated by numbers. One system then further labels genes based on whether they are transcribed in the viral sense (V; viral or packaged strand) or in the complementary sense (C). The other system simply labels genes based on their leftward (L) or rightward (R) orientation on the circular genome. For this thesis, the R and L system will be used. Examples of a bipartite Begomovirus (Tomato golden mosaic virus, or TGMV) and a monopartite Curtovirus (Beet curly top virus, or BCTV) are shown in Figure 1.1. As seen, the genome organization of a monopartite virus is almost identical to that of a bipartite A genome. In addition to the primary genome, it also was recently shown that some monopartite geminiviruses support
a satellite DNA known as DNAβ. Though DNAβ cannot replicate on its own, its presence can significantly increase the virulence of certain geminiviruses (Cui et al., 2004; Stanley, 2004).

All geminiviruses have an intergenic region (IR) that contains the origin of replication and divergent promoters. In addition, in bipartite viruses a portion of the IR called the common region (CR) is shared by the two genome components. The origin of replication includes a highly conserved hairpin loop, which all geminiviruses possess (Lazarowitz, 1987). It also contains a binding site for the viral AL1 protein (see below). A portion of the origin, including the AL1 binding site, overlaps the leftward promoter responsible for transcription of AL1, AL2 and AL3 genes. The rightward promoter is responsible for transcription of the coat protein (CP) gene, also known as AR1. The C/R repression site, located near the CP promoter, plays a vital role in the transcription of viral genes (Sunter and Bisaro, 2003). This site is bound by a putative cellular repressor, and one of the AL2 protein’s many functions is to remove the repressor bound to the CP promoter, thus contributing to CP promoter activation (Sunter and Bisaro, 2003).

1.1.2 Geminivirus replication biology

Geminiviruses are small viruses with relatively few ORFs, none of which encode polymerases. So how then do geminiviruses replicate? They must make use of the cell’s DNA replication machinery. To do this geminiviruses must activate cellular replication machinery regardless of stage in the cell cycle, redirect that machinery to the viral genome, and initiate replication. Geminivirus replication and transcription occurs via
double stranded DNA (dsDNA) intermediates in the nuclei of host cells, which is then nicked and “spun out” into single stranded copies via a mechanism called rolling circle replication (RCR) (Gutierrez, 1999; Hanley-Bowdoin et al., 2000; Stenger et al., 1991). Briefly, RCR occurs in two discrete steps. First, the single stranded (+) genome is used as a template to synthesize a negative strand (-). This double-stranded form of the genome is then used to synthesize (+) strand in a circle around the negative strand, forming a single, continuous strand of new genome. In addition to replication, the double-stranded intermediate also is the template for transcription, and associates with histones to form minichromosomes (Pilartz and Jeske, 1992; Pilartz and Jeske, 2003). As will be later discussed, this association with histones and formation of minichromosomes provides an opportunity for plants to defend against the viral genomes by DNA silencing. The geminivirus replication cycle is illustrated in Figure 1.2.

1.1.3 Geminivirus gene functions

Using TGMV, a bipartite begomovirus, as a model, the gene products of a typical geminivirus can be examined. TGMV encodes five genes on the A genome: four on the complementary strand (termed AL, or ‘left’ of the common region) and one on the virion sense strand (termed AR, or ‘right’ of the common region). The B genome contains two genes, one to the right of the common region (BR) and one to the left (BL). The AL1 or Rep protein (for Replication initiator protein) has three primary functions. The first is the conditioning of the host cell for DNA replication and recruitment of the replication machinery, for which AL1 is both necessary and sufficient (Egelkrout et al., 2001;
To do this, AL1 binds and inactivates retinoblastoma protein (Rb) which results in progression to S phase, making the host replication machinery available for virus replication (Hanley-Bowdoin et al., 2004). Second, AL1 specifically binds to the viral origin of replication in the CR, where it acts as an origin recognition protein to recruit host replication machinery. Third, it makes a specific nick in the viral strand within the loop of the conserved hairpin, which provides the primer for initiation of DNA synthesis. (Bisaro, 1996; Hanley-Bowdoin et al., 2004; Lazarowitz et al., 1992). The AL2 or TrAP protein (Transcriptional Activator Protein) is a small multifunctional protein with a variety of functions, including interaction with and inactivation of the cellular proteins SNF1 and ADK, activation of transcription, and suppression of gene silencing (Hao et al., 2003; Sunter and Bisaro, 1997; Wang et al., 2005; Wang et al., 2003). It will be described in much greater detail later in this section. The AL3, or Replication Enhancer protein (REn) is needed for optimal replication, although its mechanism remains unclear. AL3 mutants typically accumulate viral DNA in significantly reduced amounts (Sunter et al., 1990). More recent work in another begomovirus, Tomato yellow leaf curl virus, suggests that AL3 may dimerize, interact with cellular factors and interact with AL1 to enhance replication by protein-protein interactions (Settlage et al., 2005). The function of AL4 in TGMV is unknown, but in Old World begomoviruses, AL4 (also called AC4) can act as a silencing suppressor by binding to single-stranded forms of small interfering RNAs (see below) (Vanitharani et al., 2005; Vanitharani et al., 2004). The AR1 protein, immediately to the right of the common region, is the coat protein (CP). CP is necessary for plant-to-plant transmission.
It is also required for spread between cells and systemic spread in geminiviruses except in the bipartite begomoviruses (Gardiner et al., 1988). The B genome encodes only two genes, BR1, which is the nuclear shuttle protein (NSP), and BL1 (MP; movement protein). Both are required for cell-to-cell movement and spread of the virus in infected plants. Although their precise roles in movement have yet to be determined, BL1 is known to associate with plasmodesmata, whereas BR1 binds ssDNA. BL1 and BR1 also interact with one another, which suggests that BR1 delivers viral genomes to BL1 for transport between cells (Rojas et al., 2005).

As a model monopartite virus, the organization of the curtovirus BCTV is very similar to that of its bipartite cousin, TGMV. For example, the L1 and L3 genes encode proteins similar in function to their begomovirus counterparts (Hormuzdi and Bisaro, 1995; Stanley et al., 1992). The L2 protein, while homologous to AL2, has a key difference: Unlike AL2, it is not transcriptionally active and it is not required for late viral gene expression. However, recent work has shown that L2 is a pathogenicity factor, and works to suppress silencing similar to AL2 (see below) (Wang et al., 2005; Wang et al., 2003). In contrast, the rightward genes appear quite different for these genera. The R1 gene of BCTV encodes the coat protein, which in this case is essential for movement, as is the R3 protein. R2 encodes a protein needed for accumulation of ssDNA (Hormuzdi and Bisaro, 1993).
1.1.4 The TGMV AL2 (TrAP) protein

As previously stated, the AL2 or TrAP protein has two primary functions: a transcriptional activator, and a viral silencing suppressor, the first of which will be covered in this section. The 15 kDa, 129 amino acid protein AL2 is found in all begomoviruses, and also is known as AC2 (for Old World virus homologues) and C2 or L2 (for monopartite virus homologues). The first function of AL2 discovered was its transcriptional activation properties (Sunter and Bisaro, 1992). AL2 transactivates the promoter in the rightward, or virion-sense, direction on both the A and B genomes, greatly increasing the level of transcription of CP (AR1) and NSP (BR1) genes (virion-sense being defined as the same polarity as the original ssDNA genome). It does this in a virus nonspecific manner: several other begomovirus AL2 or AC2 proteins can complement a TGMV AL2 mutant (Sunter et al., 1994). AL2 mutants are not infectious, since they cannot produce either CP or NSP in significant amounts. As expected, it was shown that AL2 mutant viruses could replicate in plant protoplasts but not in whole plants (Sunter and Bisaro, 1991; Sunter et al., 1990).

How does the AL2 protein activate transcription? All transcription factors have at least one of two major domains. One is responsible for targeting the protein to responsive promoters, often by DNA-binding to specific sequences, while the other is involved in activating transcription. At the C-terminal end of AL2 is an acidic region, which further mutational studies showed to contain the minimal activation domain. A fragment as small as AL2_{115-129} showed very significant transcriptional activity, identifying the minimal activation domain as being contained within those final amino
acids (Hartitz et al., 1999). The AL2 activation domain retains its activity across kingdoms, as it is functional in plant, mammalian and yeast cells (Hartitz et al., 1999). AL2\textsubscript{1-83}, AL2\textsubscript{1-110}, and AL2\textsubscript{1-114} mutants have all been used as transcriptionally inactive mutants because all lack the minimal activation domain. In this thesis, studies utilizing transcriptionally inactive AL2 mutants utilized the AL2\textsubscript{1-114} mutant.

How AL2 is targeted to responsive promoters is still unclear. Most transcription factors are targeted by DNA binding domains that recognize specific sequence motifs located in gene promoters. However, while AL2 has been shown to have both ssDNA and weak dsDNA binding activity, these are not sequence-specific and the function of DNA binding is not known (Hartitz et al., 1999). Instead, the available evidence suggests that AL2 is targeted by interaction with a host protein(s) that recognizes specific sequences in the viral CP (and NSP) promoter. In the CP promoter this factor is believed to be a repressor (C/R protein) that binds to the C/R site (Sunter and Bisaro, 2003). Thus AL2 both derepresses the CP promoter by binding C/R, and causes transcription activation via its activation domain. Because of this mode of targeting, it is likely that AL2 can also activate cellular genes, and it has been demonstrated that this can in fact occur (Sunter and Bisaro, 1997). A related AC2 was also recently shown to cause an increase in transcription \textit{in vivo} to many native genes by microarray analysis (Trinks et al., 2005).

AL2 in addition has a zinc finger-like CCHC domain and can bind zinc (Hartitz et al., 1999). However, this domain is not needed for DNA binding activity (van Wezel et al., 2003). More recently, it has been shown that cysteine residues within the CCHC
motif are required for AL2 to dimerize, and that dimerization is required for optimal transcription activation (Yang et al., 2007). For example, a C→A mutation at amino acid 33 within the zinc finger-like motif showed partial transcriptional activity (Yang et al., 2007). In this thesis, the AL2-C33A mutant was used as a partially transcriptionally active form of AL2. However, the same mutations that attenuate transcription activity do not affect the ability of AL2 to interact with cellular kinases, including adenosine kinase (ADK) and SNF1-related kinase (SnRK1) (Yang et al., 2007).

Consistent with its transcriptional activation ability, AL2 was found to localize to the nucleus (Dong et al., 2003). However, another study showed that AL2 localized to the cytoplasm as well, and this has been confirmed by work showing that dimeric AL2 moves to the nucleus, while monomeric form interacts with adenosine kinase (ADK) in the cytoplasm (Wang et al., 2003; Yang et al., 2007).

The first indication of AL2’s second function came when transgenic N. benthamiana plants expressing AL21-100 and L2 were studied. Plants expressing those proteins showed significantly enhanced sensitivity (ES), with their ID$_{50}$ reduced by as much as 60-fold (Sunter et al., 2001). This suggested that AL2 suppressed plant defenses against viruses, and in a transcriptional activation-independent manner, since both the inactive mutant AL21-100 and L2 both showed ES. But what defense pathways might AL2 be suppressing? The next sections will explore the mechanisms plants employ to defend against pathogens, and how viral proteins such as AL2 act as counterdefensive agents to suppress these pathways.
1.2 Plant defenses against pathogens

1.2.1 Plant innate defenses: R-proteins and the hypersensitive response

In plants, the first lines of defense against invading pathogens are passive defenses, such as preformed surface wax coatings and the cell walls, which provide a physical barrier against infection. When a potential infection begins, pathogens first reveal themselves to plant innate defenses by means of elicitor molecules. These can come from the plant itself. For example, cell wall degradation products, such as oligogalacturonides, can trigger a defense response (Shibuya and Minami, 2001). However, most effector molecules come from the pathogen. Surface elements of the pathogen, such as fungal chitin, bacterial lipopolysaccharides, and flagellin elicit can elicit defense responses from a wide range of plants (Jones and Takemoto, 2004; Montesano et al., 2003). However, the largest category of elicitors from pathogens is known as the Avr or Avirulence protein family. These encompass a wide variety of pathogen effector proteins, usually proteins which the pathogen must use for infection or survival. Examples of these are the Type III effector proteins secreted by Gram negative bacteria *Pseudomonas syringae*, such as the AvrB and AvrRpmI proteins. Both of these interact with the RIN1 protein in *Arabidopsis* and can trigger a host response in strains resistant to this bacterium (da Cunha et al., 2006). Other examples include bacterial cold shock proteins and elongation factor Tu (da Cunha et al., 2006). Plant innate defenses are triggered when an elicitor protein binds to one of a large family of pattern-recognition proteins known as R-proteins. Nearly all share structural features such as Leucine-Rich
Repeats (LRRs) and Nucleotide-Binding Sites (NBSs) (Martin et al., 2003), and are similar to the Toll-like receptors that help drive mammalian innate immunity (Kopp and Medzhitov, 2003). The diversity of R-proteins is enormous; in Arabidopsis there are 149 distinct NBS-LRR proteins, compared to 23 for humans (Jones and Takemoto, 2004; Meyers et al., 2003). In contrast to mammals, which possess a mobile, flexible somatically-generated adaptive immune system, the R-proteins are often a plant’s primary line of molecular defense, so greater diversity would be expected.

The R-genes and their avr protein partners form the backbone of plant innate resistance; if either R-gene or avr is absent from a given pair, a successful infection results. More recently, however, the “guard” hypothesis proposed that the majority of R-protein recognition of effectors is done indirectly instead. The hypothesis states that the R-protein actually detects changes in the protein targets of avr proteins; when they are perturbed, the R-protein is activated and resistance is triggered (da Cunha et al., 2006).

Once a pathogen is detected, a diversity of innate defense responses are triggered. The initial steps include a Ca$^{2+}$ boost in the cytosol, an oxidative burst, kinase cascades and nitrous oxide (NO) production (da Cunha et al., 2006). In addition, plants produce defensive hormones such as salicylic acid, jasmonic acid, and ethylene (Kunkel and Brooks, 2002). The end result of a successful defensive response is usually the Hypersensitive Response (HR), a form of localized, programmed cell death which contains and eliminates the pathogen, preventing its spread to the surrounding tissue (Bent, 1996; Morel and Dangl, 1997). The interaction between a potential pathogen and
the plant innate defensive responses can be thought of as a continuum, with disease or the hypersensitive response being two primary outcomes.

1.2.2 A plant adaptive defense: RNA silencing

In addition to the innate defenses, plants also have the ability to defend against intracellular pathogens by a pathway known alternatively as RNA silencing, Post Transcriptional Gene Silencing (PTGS), or quelling. In addition to its role in plant defenses, RNA silencing pathways are fundamental to plant metabolism. Common players in all the RNA-silencing pathways include the families of the ribonuclease Dicer-like proteins (DCL), RNA-dependent RNA polymerase (RDR) and Argonaute proteins (AGO). As in the NBS-LRR R-proteins, there is a much larger diversity of Dicer and AGO proteins in plants than in mammals: the *Arabidopsis* genome encodes four DCLs and 10 AGO proteins, compared to a single Dicer and four AGO proteins in humans (Vazquez, 2006). The functions of the DCL proteins are partially redundant, but still have distinct functions within the small RNA pathways (Blevins et al., 2006; Gasciolli et al., 2005; Henderson et al., 2006). Several native small RNA driven pathways have been characterized. The first and most well-characterized involves microRNAs (miRNAs), which are often tissue-specific and primarily encompass developmental control. Cellular miRNA genes encode hairpin-containing primary miRNAs, which are processed in the nucleus by the ribonuclease Drosha into smaller pre-miRNAs. These move to the cytoplasm where they are cleaved by DCL1 into 21-22 nt miRNAs, which are similar to small interfering RNAs (siRNAs; see below). It appears that miRNA processing is
accomplished almost exclusively through DCL1; dcl1 null mutants are embryonic lethal and knockdown mutants exhibit severe developmental defects (Gasciollli et al., 2005; Kurihara and Watanabe, 2004; Schauer et al., 2002). More recently, it has been even been suggested that miRNAs may provide another layer of innate defense against viruses. Recombinant viruses are briefly blocked by miRNAs homologous to portions of their genomes (Lu et al., 2008; Simon-Mateo and Garcia, 2006), and there are a number of homologies between Arabidopsis small RNAs and viral genomes (Llave, 2004). However, actual experimental evidence of miRNAs blocking viral infection is sketchy. Perhaps more importantly, it has recently become clear that at least some mammalian viruses, for example herpes simplex virus 1, encodes miRNAs in order to regulate their own genes, and possibly also host genes. Evidence that infection with herpesviruses can alter cellular miRNA profiles has also been obtained, although the significance of this is not yet clear.

In addition to the miRNAs, several other native small RNA pathways have been identified in plants, encompassing functions as diverse as gene regulation (trans-acting siRNAs and native-siRNAs), heterochromatin formation, and RNA-directed DNA methylation (repeat-generated siRNAs, native-siRNAs) (Baulcombe, 2004; Carrington and Ambros, 2003; Vaucheret, 2006; Vazquez, 2006).

However, the primary adaptive defense against intracellular pathogens in plants is the post-transcriptional gene silencing (PTGS), or exogenous siRNA pathway. It was the first RNA silencing pathway identified (van der Krol et al., 1990). The archetypical trigger is a double-stranded RNA species, such as those produced by RNA viruses
However, it can also be triggered by overabundant or “aberrant” RNA such as produced by transgenes (Dalmay et al., 2000; Gazzani et al., 2004; Mourrain et al., 2000). Both pathways are illustrated in Figure 1.3 (Brodersen and Voinnet, 2006). Briefly, a template (such as an RNA virus ds replication intermediate or RNA hairpin) is detected and processed into 21-22 or 24 nt dsRNA species by DCL2/DCL4 or DCL3 proteins, respectively. Small interfering RNAs (siRNAs) are short dsRNA duplexes with 2 nt 3’-OH overhangs. These siRNAs (and miRNAs) are subsequently methylated by the HEN1 protein to enhance stability, separated into single-stranded siRNA by a helicase such as SDE3 (Dalmay et al., 2001), and incorporated into a ribonucleoprotein complex known as RISC (RNA-induced silencing complex). A key component of the RISC complex is AGO1. AGO1 is a ribonuclease slicer protein that, in turn, targets genomic and mRNAs with homologous sequences to the siRNA and cleaves them into more small RNAs (Baumberger and Baulcombe, 2005). As well as being incorporated into the RISC complexes, the resulting siRNA can also be amplified by means of the RDR proteins (Ding and Voinnet, 2007; Himber et al., 2003). The most well characterized RDR of the pathway is RDR6, which is further described below.

1.2.2.1. PTGS: local vs. systemic silencing

One of the more remarkable aspects of PTGS is that it operates at three different levels. The first is at the target cell level, where initial infection results in the production of 21-22 and 24 nt RNAs as noted above. The second is the short-range cell-to-cell
transmission of the silencing signal. Indirect evidence indicates the spread of the short-
range silencing signal takes place through the plasmodesmata, based on the findings that
the guard cells of stomata, which do not have plasmodesmata, escape silencing during
short range spread (Himber et al., 2003; Kalantidis et al., 2006). The 21-22 nt siRNA is
apparently responsible for this cell-to-cell spread, as the ability of silencing to spread
locally is lost in *dcl4* mutants in *Arabidopsis* (Dunoyer et al., 2005). Recent work also
implicates the *NRPD1a* and *RDR2* genes, both associated with DNA-dependent RNA-
polymerase IV, as being required for the cell to cell spread of silencing, though their role
remains unclear (Kalantidis et al., 2008).

The third level, and one most closely studied in this thesis, is the cell-autonomous
or systemic spread of the silencing signal. In systemic silencing, unlike the cell to cell
transmission, a silencing signal generated at the source travels through the vascular
system of the plant and induces silencing in sink leaves, spreading gradually downward
until the entire plant is silenced. Systemic silencing was first demonstrated in *N. tabacum*
and *N. benthamiana*, by grafting and agroinfiltration experiments against a GFP
transgene, respectively (Palauqui et al., 1997; Voinnet and Baulcombe, 1997). A great
unknown of systemic silencing is the exact nature of the silencing signal. Data strongly
suggests, however, that there is a small RNA component. Silencing suppression proteins
such as p19 and HC-Pro that sequester small RNAs, for example, efficiently block the
spread of systemic silencing (Hamilton et al., 2002). Another clue comes from the RNA-
dependent RNA polymerase RDR6. When the transgenic RDR6-knockdown *N.
benthamiana* line NbRDR6 was created, scions of the *RDR6i* plant, when fused to a
silenced, wild-type rootstock, did not develop systemic silencing. However, when a
systemic silencing trigger was applied to an RDR6i rootstock, which then was fused to an
unsilenced WT scion, systemic silencing developed in the scion. This showed that RDR6
was necessary for the response to but not the generation of the systemic silencing signal
(Schwach et al., 2005). In addition, in Arabidopsis, rdr6 mutants could not sustain the
spread of systemic silencing beyond 10-15 cells past the signal’s original point, and
lacked secondary siRNAs in the unsilenced tissues (Himber et al., 2003; Voinnet, 2005b).
From the origin side, it was shown that DCL4 was required for cell to cell spread of a
silencing signal and for systemic spread of viral infection, producing a 21-nt small RNA
species (Deleris et al., 2006; Dunoyer et al., 2005). It was also suggested that those 21-nt
species may be what RDR6 uses for cell to cell amplification, both short and long
distance spread of the signal (Dunoyer et al., 2005). One possible model for how
systemic silencing might take place is illustrated in Figure 1.4 (Voinnet, 2005b). RNA
or ribonucleoprotein “transmitters” (possibly derived from degraded target mRNA) exit
the origin cell through the plasmodesmata. They enter the phloem and travel to sink
tissue, where they enter the target cell as a ssRNA species. This in turn is made into
dsRNA by RDR6, and becomes a new substrate for DCL4. The resulting 21-22nt
siRNAs can then themselves be amplified and spread. Systemic silencing can also be a
defense against viral spread through the plant; knocking down RDR6 in N. benthamiana
makes the plants hypersusceptable to Potato virus X (PVX), Potato virus Y (PVY) and
the Y satellite of Cucumber mosaic virus (CMV), although not all viruses tested had
enhanced infections in the RDR6i line (Schwach et al., 2005). This notion is further
supported by data showing the CMV protein 2b could specifically suppress systemic silencing (Guo and Ding, 2002). This thesis also provides evidence that a DNA virus-produced silencing suppressor, the geminiviral protein AL2, can also suppress systemic silencing. In addition to 2b and AL2, there are many, many more virus silencing suppressors that act upon many portions of the PTGS pathway, both local and systemic, which be explored in greater detail below.

1.2.2.2. PTGS maintenance

A curious absence from the material published is any hypothesis as to how systemic PTGS is maintained. It has long been shown that systemic silencing is a reversible process; the African cassava mosaic virus (ACMV) AC2 (AL2) expressed in a PVX vector is capable of reversing systemic transgene silencing (Voinnet et al., 1999). This was quickly followed by evidence showing that another silencing suppressor from the potyvirus Tobacco etch virus (TEV) helper-component protease (HC-Pro), targets a maintenance step by reversing established silencing of a β-glucuronidase transgene. Although little supposition was made as to the nature of the maintenance pathway which HC-Pro was interacting with, one possible clue came from the fact that the target gene coding region was partially demethylated in the presence of HC-Pro (Llave et al., 2000). Nevertheless little is still known about how systemic PTGS is maintained. One of the results detailed further in this thesis, however, suggests that there may be more then one maintenance pathway. One operates during vegetative growth while the other is active after flowering.
1.3 Plant Epigenetics and Transcriptional Gene Silencing (TGS)

1.3.1 General DNA-methylation characteristics in plants

In addition to the primary sequence of genes, there is an additional layer of heritable information in the form of cytosines methylated at the 5th position of their pyrimidine ring (5-methylcytosine), and in the form of covalently modified histones. This additional layer of regulation is termed epigenetic, and can influence the structure and activity of genes by altering the state of the chromatin (active euchromatin or methylated, silenced heterochromatin) rather than the primary sequence information. Within the animal kingdom, the 5-methylcytosine modification is limited only to the CG context, whereas in plants, methylation can take place in CG, CNG (where N = A, T, C, or G) and CHH or “asymmetric” contexts (where H = A, T, or C). In the plants examined in detail, methylation is present throughout the genome, but concentrated around the centromeres. Shotgun bisulfite sequencing showed the percentage in Arabidopsis of CG sites methylated is about 25%, CNG sites ~6.7%, and CHH sites ~1.7%, underscoring that each methylation context has differing maintenance requirements (Cokus et al., 2008; Lister et al., 2008).

In higher eukaryotes, DNA methylation is involved in numerous essential processes and most importantly is involved in transcriptional gene silencing (TGS). In plants, a key function of DNA methylation is to suppress transposon mobility (Bender,
In addition, there is extensive methylation in repeat-rich areas such as the centromere and ribosomal RNA-encoding regions, which may help prevent uncontrolled recombination and rearrangement (Cokus et al., 2008; Lister et al., 2008; Zhang, 2008). There is even recent evidence to show that DNA methylation is important for regulating the transcription of some native genes (Zhang et al., 2006; Zilberman et al., 2007). DNA methylation is also critically important for development. For example, null mutations in mouse cytosine methyltransferase genes result in embryonic lethality (Goll and Bestor, 2005). In *Arabidopsis*, mutations in methyltransferases controlling either CG or non-CG methylation results in severe developmental abnormalities (Chan et al., 2006; Mathieu et al., 2007; Saze et al., 2003). Abnormal methylation also has recently been implicated in cancer in humans, showing the importance of epigenetics in mammals (Jones and Baylin, 2002; Plass, 2002).

As mentioned above, three distinct methylation maintenance pathways have been characterized in *Arabidopsis*. Different cytosine methyltransferases are responsible for methylation in different sequence contexts, although there is considerable redundancy of function. The first involves the mammalian DMNT1 homologue MET1 (METHYLTRANSFERASE1), which primarily maintains CG methylation (Finnegan et al., 1996). The frequently studied *met1* mutant in *Arabidopsis* shows an almost complete abolition of CG methylation, though other methylation contexts remain largely untouched (Cokus et al., 2008; Lister et al., 2008; Saze et al., 2003). MET1 orthologs also has been found in other plants, and it has been shown in *N. benthamiana* that the silencing of
MET1 causes developmental abnormalities and reverses transcriptional gene silencing (Jones et al., 2001). CHROMOMETHYLASE3 (CMT3) is primarily responsible for maintenance of CNG methylation, in conjunction with proteins that methylate histone H3 at lysine 9 (H3K9) and lysine 27 (H3K27) (Bartee et al., 2001; Jackson et al., 2002; Lindroth et al., 2001; Lindroth et al., 2004). The DOMAINS REARRANGED METHYLASE 1 and 2 (DRM1/2) proteins are mammalian DNMT3 homologues thought to be responsible for all de novo cytosine methylation, and also to maintain DNA methylation at asymmetric (CHH) sites (Cao and Jacobsen, 2002a; Cao and Jacobsen, 2002b).

1.3.1.1. Significance of different sequence contexts

Recently, several attempts have been made to construct a DNA methylation map of the entire Arabidopsis genome (Cokus et al., 2008; Lister et al., 2008; Zhang et al., 2006). One of the more interesting questions to come from this work is the significance, if any, of the different methylation contexts within the genome. Are they localized to specific portions of the genome? Do they target different kinds of genes, pseudogenes, or repeat regions? Differences had been previously found, as the prototype promoter FWA was largely CG methylated (Saze et al., 2003), the small retrotransposon AtSN1 had both CG- and non-CG methylation (Zhang et al., 2006; Zilberman et al., 2003), and the transposon Ta3 was exclusively non-CG methylated (Cao and Jacobsen, 2002a; Zhang et al., 2006). However, the study by Zhang et al showed that in met1 mutants there was very significant reactivation of transposons and pseudogenes, suggesting that CG-
methylation alone was enough to account for silencing in these genes. Transcriptionally-silenced (promoter-methylated) genes were also preferentially expressed in the met1 mutant background. So what is the significance of non-CG methylation? In the ddc mutant, deficient for all non-CG methylation, a large number of known genes had their transcriptional silencing reversed, in contrast to the met1 mutants, whose transcript profile largely showed an increase in transcription of pseudogenes (Zhang et al., 2006). In addition, met1, drm1/2, and cmt3 mutant Arabidopsis showed significantly more severe symptoms when infected with the geminiviruses Cabbage leaf curl virus (CaLCuV) and BCTV than wild-type plants, suggesting that both CG- and non-CG methylation play a part in viral defense (Raja et al., 2008).

1.3.2 TGS and RNA-directed DNA methylation (RdDM)

There are two main forms of transcriptional gene silencing, or TGS. The first refers to the silencing of a normal coding gene at the transcriptional level, usually by methylation within its promoter region. The second refers to silencing found in the heavily methylated regions of the genome known as heterochromatin, including repeat sequence and pseudogenes that may be methylated throughout their length, not just within promoters. Many native genes and pseudogenes in plants such as Arabidopsis are transcriptionally silenced in one form or another, and as described above, several become active in methylation-defective mutants (Yamada et al., 2003; Zhang et al., 2006). The pathway that seems to link both of these forms of TGS is RNA-dependent DNA methylation, or RdDM. The first indications of this phenomenon came from studies of a
plant RNA viroid (Wassenegger et al., 1994). Following that initial discovery it was found that the 24-nt class of siRNAs was associated with the Tnt1 and SINE element retrotransposons, and an *Arabidopsis* mutant that abolished the AtSN1 transposon-specific RNA also abolished methylation in that transposon (Hamilton et al., 2002). More siRNAs corresponding to several endogenously silenced loci, such as retrotransposons, centromeric repeats, and rDNA were then discovered (Chan et al., 2005). More recently, it was found that a large subset of native siRNAs are associated with heterochromatic DNA and target DNA through RdDM (Lister et al., 2008; Qi et al., 2006; Zhang et al., 2006). These small RNAs tracked the methylation state of their target sites closely; in the *met1* mutant background, both the methylation and number of small RNAs at many loci dropped precipitously, while in the *rdd* mutant (deficient in demethylation activity), some unmethylated loci became hypermethylated, with a corresponding increase in siRNAs around the newly methylated regions (Lister et al., 2008). It is even possible to artificially trigger TGS against a transgene by using virus-induced gene silencing (VIGS) to cause promoter methylation (Jones et al., 2001), a technique used in this thesis. Small RNAs are thus closely tied to DNA methylation, but it is unclear how they work.

Several similar models exist to explain the mechanism of the heterochromatin siRNA – DNA methylation feedback cycle (Brodersen and Voinnet, 2006; Chapman and Carrington, 2007; Vaucheret, 2006). One commonality, and a key component, is the protein ARGONAUTE4 (AGO4). AGO4 was first described as important to methylation in a study of the *ago4-1* mutant in which the methylated loci SUPERMAN locus and
AtSN1 lost significant methylation of CNG and CHH sites, although CG sites were only mildly perturbed. There was also a complete loss of AtSN1 small RNAs in ago4-1 mutants (Zilberman et al., 2003). Other research then showed that AGO4 was required for the maintenance, but not the generation, of siRNA from inverted repeats, was a nuclear protein localized to nuclear Cajal bodies, and partnered with the enzyme RNA PolIV, possibly linking AGO4 with transcription from methylated templates (Herr et al., 2005; Li et al., 2006; Onodera et al., 2005; Till and Ladurner, 2007; Zilberman et al., 2004). More recent research has helped to clarify the role of PolIVa and PolIVb in TGS and other kinds of gene silencing. PolIV has been shown to be necessary for siRNA generation at most endogenous loci, and may participate in all four DCL-mediated silencing pathways (Mosher et al., 2008; Pikaard et al., 2008), although the exact role PolIVa and b may play remains to be seen. Like PolIV, the exact role of AGO4 in RdDM remains unclear. One model for the RdDM pathway in plants suggests that target sequences are transcribed by PolIVa, and subsequently converted to dsRNA by complexes containing RDR2. DCL3 then processes 24 nt siRNAs from this dsRNA, and the siRNAs are loaded into an AGO4 complex that associates with PolIVb. The AGO4-associated siRNAs target the complex to homologous DNA sequences, where cytosine methyltransferases are recruited (Brodersen and Voinnet, 2006).

TGS is clearly vital to the plant cell in regulating its genes, chromatin remodeling and maintenance of heterochromatin. But might it also play a role, as PTGS does, in plant defense against viruses?
1.3.3 Can TGS be a defense against plant viruses?

We hypothesized that TGS might also be a defense against DNA viruses as their double-stranded replicative intermediates in the nucleus would be subject to methylation, histone modification and possibly RdDM. Recently, a study was completed by our lab that showed several lines of evidence that DNA methylation and histone modification were indeed employed as a defense against geminiviruses (Raja et al., 2008). First, we did infection studies with CaLCuV and BCTV in several mutant lines of Arabidopsis deficient for various enzymes for methylation and small RNA metabolism. As previously stated, met1 mutants showed some symptom enhancement while drm1/2 and cmt3 mutants showed much more severe symptoms. kyp2 mutants (H3K9 methyltransferase) likewise showed severe symptoms, suggesting a role for histone methylation in defense as well. Other methylation pathway component mutants (ago4, nrpd2a, and ddm1) also showed significant enhancement of symptoms. Finally, as predicted by previous work with TGMV AL2 (Wang et al., 2005; Wang et al., 2003), adk1 and adk2 mutants showed significant enhancement of symptoms, confirming the importance of the methyl cycle enzyme ADK in defense against geminiviruses. Next, bisulfite sequencing was carried out with BCTV and CaLCuV DNA from infected plants, and revealed a very significant amount of methylation in the IR. In methylation-deficient mutants, several hypermethylated sites become hypo- or even unmethylated as well on both viral genomes. Next, as kyp2 mutant showed increased sensitivity, chromatin immunoprecipitation (ChIP) analysis was done on the CaLCuV IR region. This study
showed that both active (acetylated H3) and repressive (H3K9) marks were present from the histones associated from the IR, suggesting that there were populations of both active and repressed genomes during an infection. Finally, and most interestingly, recovery studies were undertaken of BCTV infection of WT and ago4 mutant Arabidopsis, using both WT BCTV and a BCTV mutant L2\(^{-}\). It was shown that though primary infection with both mutant and WT BCTV proceeded similarly, after removal of primary shoots, BCTV L2\(^{-}\) plants recovered and became almost symptom free, and the little BCTV isolated from the recovered tissue was hypermethylated. However, ago4 mutant plants could not recover, as both WT and mutant BCTV showed severe symptoms in ago4 plants along with a much lower level of methylation -- more evidence that RdDM played a role in defense against these viruses, and that the L2 protein was the probable suppressor of this defense pathway. Overall, the study showed that TGS and DNA methylation could indeed be a defense against DNA viruses, and it also set the stage for much of the research described in this thesis, to show specifically that the geminiviral proteins AL2 and L2 could suppress both PTGS and TGS-related host defenses.

1.4 Plant virus counterdefenses

1.4.1 Plant RNA virus silencing suppressors

To counter the highly effective RNA-silencing pathway defenses that plants possess, many DNA and RNA plant viruses encode a suppressor of RNA silencing (Ding and Voinnet, 2007; Kalantidis et al., 2008; Li and Ding, 2006). The first viral silencing
Suppressor identified was the Helper Component Protease (HC-Pro) from *Tobacco etch virus* and other potyviruses. It was one of the first proteins shown to reverse established systemic silencing (Brigneti et al., 1998; Llave et al., 2000), and it also suppresses both transgene-induced silencing and VIGS (Anandalakshmi et al., 1998; Brigneti et al., 1998). Owing to its ability to reverse systemic PTGS, it was used as a positive control for some of the work in this thesis. Another well-studied silencing suppressor is the p19 protein from *Tomato bushy stunt virus* and other tombusviruses. It was first identified for it’s ability to suppress systemic spread and suppress transgene-mediated gene silencing, and very early was shown to bind and sequester small RNAs (Qu and Morris, 2002; Silhavy et al., 2002). It is an extremely potent silencing suppressor, significantly enhancing gene expression in a transient system when co-expressed (Voinnet et al., 2003). P19 even works between kingdoms, being an active RNA silencing suppressor in animal systems as well (Voinnet et al., 1999). Owing to its ability to bind small RNA, it also is an effective suppressor of systemic PTGS, though it cannot reverse established systemic PTGS (Silhavy et al., 2002). Thus p19 is an effective positive control for both local silencing and systemic silencing suppression assays, covered in this thesis. Other RNA virus silencing suppressors of interest include the *Cucumber mosaic virus* protein 2b, which can suppress systemic silencing but not local silencing (Brigneti et al., 1998; Guo and Ding, 2002), and the *Turnip crinkle virus* coat protein (CP), which blocks both local and systemic silencing by interacting with and suppressing the activity of DCL-2 (Qu et al., 2003).
1.4.2 Geminivirus silencing suppressors

Like RNA viruses, plant DNA viruses are also known to encode an array of silencing suppressors. Among the geminiviruses, there are three primary silencing suppressors known. They are the AC2/AL2/TrAP protein, the AC4 protein, and the βC1 satellite DNA protein. The earliest of the geminivirus proteins identified as a suppressor of silencing was *African cassava mosaic virus* AC2 (AL2), shown by Voinnet *et al* to be capable of reversing established silencing of a transgene when expressed from a PVX vector (Voinnet *et al*, 1999). The next studied was the C2 (AL2) protein of the monopartite *Tomato yellow leaf curl virus*, which was shown to require the zinc finger domain in order to suppress silencing (van Wezel *et al*, 2002). Further studies with the AC2 (AL2) protein from *Mung bean yellow mosaic virus* (MBYMV) indicated that transcriptional activity was required for suppression (Trinks *et al*, 2005). An intriguing aspect of that study were microarray experiments which showed that in *Arabidopsis* protoplasts, AC2 expression led to the increase in expression of more than 30 host genes. One of these, *WEL1*, may be a negative regulator of PTGS (Trinks *et al*, 2005). The AL2 gene from *Tomato golden mosaic virus* and the L2 gene from *Beet curly top virus* are also extensively studied silencing suppressors; they are described in more detail below.

A more recent addition to the family of geminiviral suppressors was the AC4 protein. The AC4 protein lies entirely within the coding region of the AC1 or Rep protein and is the least conserved of all the geminiviral proteins, both in sequence and in function. Some AC4 mutants have no phenotype, whereas other AC4s can act as
symptom determinants and improve infectivity (Krake et al., 1998; Latham et al., 1997).  
A study of several AC2 and AC4 proteins showed that the AC4 of *Sri Lankan cassava mosaic virus* and *ACMV* (Cameroon) both suppressed local silencing in an agroinfiltration assay (Vanitharani et al., 2004).  AC4 also caused developmental defects when expressed as a transgene in *Arabidopsis*, and suppressed the production of the microRNA miR159.  It also was shown to bind short ssRNA by co-precipitation of protein/miRNA complexes (Chellappan et al., 2005).  This suggests that ACMV AC4 acts by a mechanism similar to p19, except that instead of sequestering siRNA duplexes, it sequesters them in single-stranded form after they are unwound.  Both mechanisms could prevent the proper assembly of RISC complexes.

Most recently, a new class of geminiviral silencing suppressors was discovered in β-satellites.  The β-satellites are a non-autonomous small ssDNA family of approximately ~1360 nucleotides, with over 260 members deposited to databases thus far (Briddon et al., 2008).  They cannot replicate on their own, but require a “host virus” to replicate and spread.  The β-satellites all encode a single gene, βC1, which can act as a pathogenicity determinant and is required for the efficient infection of some hosts (Briddon et al., 2001; Saeed et al., 2005).  βC1 is a suppressor of PTGS that localizes to the cell nucleus (Cui et al., 2005).  Although the mechanism of action of βC1 is unknown, it has been speculated that it may target a step in the silencing pathway that overlaps with miRNA generation, owing to its ability to cause severe developmental defects when expressed in PVX or as a transgene (Cui et al., 2004; Saeed et al., 2005;
Other studies have suggested that βC1 may also be involved in virus movement (Briddon et al., 2008).

1.4.3 The silencing suppressors AL2 and L2

As already described, it has been known that some AC2 and C2 (AL2) proteins of the Old World begomoviruses can act as silencing suppressors, but recently we have shown that AL2 from New World begomoviruses and L2 from curtoviruses also have this ability. Both *Tomato golden mosaic virus* (TGMV) AL2 and the *Beet curly top virus* protein L2 suppress local PTGS (Wang et al., 2005). The first indication that they might serve as pathogenicity factors came in a study that showed that transgenic *N. benthamiana* plants expressing transcriptionally inactive AL2\textsubscript{1-110} and L2 proteins were more susceptible to a wide range of viruses, which was attributable to their ability to inactivate the metabolic regulatory protein SNF1 kinase (Hao et al., 2003; Sunter et al., 2001). Both AL2 and L2 were then shown to interact with and inactivate two proteins: the metabolic switch protein SNF1 and adenosine kinase (ADK), an important protein in the methyl cycle (Figure 1.5) (Wang et al., 2005; Wang et al., 2003). A important consequence is that both kinases are inactivated by AL2 and L2. A primary function of ADK is to scavenge adenosine by converting it into AMP. In the methyl cycle, ADK acts to remove adenosine from the reaction catalyzed by S-adenosyl homocysteine hydrolase (SAHH), which generates adenosine as a waste product and is required for the production of S-adenosyl methionine (SAM), a methyl donor and essential cofactor for most methyltransferase reactions. Studies have shown that ADK is vital in maintaining DNA
methylation, and that knocking down ADK expression results in severe developmental abnormalities and a reduction in total cellular methylation (Moffatt et al., 2002; Schoor and Moffatt, 2004; Weretilnyk et al., 2001). Knocking down ADK in a transient agroinfiltration assay was also shown to suppress PTGS (Wang et al., 2005). As would be predicted by its importance in DNA methylation, ADK was also recently shown to be required for TGS as well (Rocha et al., 2005).

There are two predicted mechanisms for AL2 suppression of silencing. The first is its interaction with ADK, which is independent of its ability to activate transcription. It was shown that the mutant AL2_{1-114} and L2, neither of which were transcriptionally active, were capable of suppressing PTGS in a local assay, and that ADK activity in the infiltration zones was significantly reduced. Consistent with the ability of AL2 and L2 to inhibit ADK, ADK knock down by RNA interference, and chemical inhibition of ADK activity with the nucleoside analogue A-134974 also suppressed PTGS in the local assay (Wang et al., 2005). But in addition to this transcription-independent mechanism, it was also predicted that AL2 may have a transcription-dependent mechanism for suppressing silencing as well, based on the observation that MBYMV AC2 requires the transcription activation domain for some aspects of silencing suppression, and that it might transactivate an endogenous silencing suppressor (Trinks et al., 2005). To date no direct proof exists that this alternate mechanism of silencing suppression involves transcriptional activation of host genes. However, for convenience, this activation-domain-dependent mechanism will be referred to as transcription-dependent or transcription-activation dependent throughout the thesis.
This thesis extends the work done by Wang et al. on the silencing suppression activities of AL2. It is first shown that AL2, in addition to suppressing local silencing, is also capable of blocking systemic silencing. Unlike local suppression, systemic silencing suppression activity is shown to be transcription-dependent, and inhibition of methyl cycle enzymes such as SAHH or ADK did not block systemic silencing. It is also shown that, like the ACMV protein AC2 (AL2), both TGMV AL2 and BCTV L2 are capable of reversing established PTGS. This suggests interference with a PTGS maintenance step. AL2 can reverse silencing in both young and mature plants, whereas L2 can only reverse silencing in young plants. More specifically, L2 is significantly less effective in reversing silencing in plants that have undergone the vegetative to floral transition, suggesting the presence of multiple, developmentally-regulated maintenance pathways for PTGS. Finally, as hypothesized, work described in this thesis shows that AL2 and L2 can also reverse TGS and cause global demethylation of cytosine residues in plants, the first such example of viral proteins capable of reversing TGS.
Figure 1.1 Geminivirus gene organization

The diagram depicts the double-stranded replicative forms of *Maize streak virus* (MSV, *Mastrevirus*), *Beet curly top virus* (BCTV, *Curtovirus*), *Tomato yellow leaf curl virus* (TYLCV, monopartite, *Begomovirus*), and *Tomato golden mosaic virus* (TGMV, A and B, bipartite, *Begomovirus*). The solid arrows indicate the positions of viral genes with the approximate molecular mass of each encoded protein given in kD. Viral genes are designated by number and the direction of transcription from the double stranded intermediate: leftward (L, complementary sense) or rightward (R, viral sense). Certain viral genes are also indicated by name, including Rep (replication initiator protein), TrAP (transcriptional activation protein), REn (replication enhancer), NSP (nuclear shuttle protein) and CP (coat or capsid protein). The position of the conserved hairpin is indicated by an asterisk within the intergenic region (IR). The common region (CR), a sequence of ~230 bp that is nearly identical in TGMV DNAs A and B, is indicated by a hatched box (Bisaro, 1996).
Figure 1.1 Geminivirus gene organization.
Figure 1.2 Geminivirus replication.

Geminivirus DNA replication occurs in two stages. First, the ssDNA is converted into dsDNA. The dsDNA, in turn, serves as a template for viral gene expression. Secondly, the dsDNA serves as a template for the rolling circle replication cycle to produce new ssDNA products. To initiate replication, Rep (AL1) binds and inactivates Rb to move cells into S phase (not shown). It also binds the origin of replication, where it recruits host replication machinery. Rolling circle begins when Rep nicks the viral strand, creating a 3’-OH terminus needed to prime DNA synthesis. After nicking, Rep binds to the 5’-end of the nick site through a tyrosine residue. Replication proceeds continuously around the intact circular template. When the origin is regenerated, Rep again nicks the nascent molecule releasing a unit length single strand which is subsequently ligated to circular form. Rep then associates with the 5’-terminus generated by the nick and the process continues. The replication enhancer protein (REn; AL3) stimulates replication in an unknown manner. Progeny ssDNA can then re-enter the DNA replication pool, associate with coat protein, or be transported outside the nucleus and to the neighboring cells with the help of the nuclear shuttle protein (NSP; BR1) and the movement protein (MP; BL1). The transcriptional activator protein (TrAP; AL2) is required for the expression of late viral genes including coat protein (CP) and MP.
Figure 1.2 Geminivirus replication.
Figure 1.3 PTGS silencing pathways [adapted from (Brodersen and Voinnet, 2006)].

(a) The IR-PTGS pathway. An inverted repeat transgene construct, typically employed for RNAi in plants, produces ds transcripts with perfectly complementary arms. Two distinct Dicer-like (DCL) enzymes process the ds transcripts. DCL3 probably produces siRNAs of the 24 nt size class, which can direct DNA or histone modification at homologous loci and which appear to be dispensable for RNA cleavage. DCL4 is probably the preferred enzyme for production of 21-nt siRNAs from the dsRNA. One siRNA strand incorporates into AGO1-loaded RISC to guide degradation of homologous RNA.

(b) The S-PTGS pathway. The pathway is shown here as being elicited by RNAs with aberrant features (such as overabundance, lacking 5’ caps, or lacking 3’ poly-A tails). Aberrant RNA is converted into dsRNAs by the combined action of RDR6, SGS3, SDE3 and possibly WEX. The resulting dsRNA is then processed by a DCL, probably DCL4, producing siRNAs that are exclusively of the 21 nt size class. These molecules can then undergo two sets of reactions. First, they can be used as primers by RDR6 to reinforce production of dsRNA from single-stranded templates. They can also incorporate into AGO1-loaded RISC to guide sequence-specific cleavage of homologous RNA as in (a). The resulting cleavage products could be perceived as aberrant RNAs and thus could promote further production of dsRNA, resulting in an amplified reaction.
Figure 1.3 PTGS silencing pathways [adapted from (Brodersen and Voinnet, 2006)].
**Figure 1.4** Possible systemic silencing mechanism. Adapted from (Voinnet, 2005b).

An illustration of a re-iterative model for cell-to-cell transgene silencing movement involving trafficking and amplification of the 21nt siRNA. The results in *N. benthamiana* and *Arabidopsis* indicate that cell-to-cell movement of transgene silencing can be resolved into short-range and long-range phases. Long-range movement would occur through the phloem and would be dependent upon RDR6 and SDE3, which use homologous transcripts as templates to produce new dsRNA. This molecule is processed into secondary siRNAs that are exclusively of the 21nt size class, the proposed nucleic acid component of the short-range signal. In this model, DCL4-dependent, 21nt-long primary siRNA produced at the site of initiation would move to 10–15 adjacent cells, independently of the presence of homologous transcripts in those cells. Movement would require the products of SMD family and other as yet unidentified cellular factors, possibly physically associated with siRNAs. Primary siRNAs could initiate, in recipient cells, the synthesis of secondary, 21nt-long siRNAs through the combined action of RDR6/SDE3 (using homologous transcripts as templates) and, possibly, of DCL4. The re-iterated short-distance signaling events would then eventually translate into extensive, long-range movement through the phloem.
Figure 1.4 Possible systemic silencing mechanism. [Adapted from (Voinnet, 2005b)].
**Figure 1.5** The methyl cycle.

SAM (S-adenosyl methionine) is the methyl donor for most transmethylation reactions. The product SAH (S-adenosyl homocysteine) is a methyltransferase inhibitor. SAH is converted to Hcy (homocysteine) and adenosine by SAHH (S-adenosyl homocysteine hydrolase). Phosphorylation of adenosine by ADK (adenosine kinase) is important because the SAHH reaction is reversible and the equilibrium lies in the direction of SAH synthesis. THF, tetrahydrofolate.
**Figure 1.5** The Methyl cycle.
CHAPTER 2

GEMINIVIRUS AL2 AND L2 PROTEINS SUPPRESS TRANSCRIPTIONAL GENE SILENCING AND CAUSE GENOME-WIDE REDUCTIONS IN CYTOSINE METHYLATION

The following chapter was submitted for publication to the Journal of Virology as follows: Buchmann, R.C., Asad, S., Mohannath, G., Bisaro, D.M. Geminivirus AL2 and L2 proteins suppress transcriptional gene silencing and cause genome-wide reductions in cytosine methylation. Figures 2.2, 2.5, and 2.7 were done by Shaheen Asad; Figure 2.3 was done in collaboration with Gireesha Mohannath, and Figure 2.8 was done in collaboration with Jamie Wolf.

2.1 Introduction

Covalent modification of eukaryotic DNA and associated histones is a fundamental epigenetic mechanism that governs the expression of selected genes and genetic elements and distinguishes heterochromatic and euchromatic regions of the genome. For example, cytosine methylation is associated with the promoters of transcriptionally silenced genes and with repressed heterochromatic sequences including...
centromeric repeats, transposons, and retroelements (Cokus et al., 2008; Lister et al., 2008). How sequences are targeted for methylation is not entirely understood but in organisms such as fission yeast and plants, small interfering RNAs (siRNAs) produced by mechanisms related to RNA interference (RNAi) guide methylation to homologous sequences in a process termed RNA-directed DNA methylation (RdDM) (Bender, 2004; Chan et al., 2005; Henderson and Jacobsen, 2007; Martienssen et al., 2005). Promoter methylation is typical of transcriptional gene silencing (TGS), although coding region methylation (body methylation) may also be present in both repressed and active genes (Cokus et al., 2008; Lippman et al., 2004; Zhang, 2008; Zhang et al., 2006). Body methylation sometimes also occurs as a consequence of post-transcriptional gene silencing (PTGS), although its role in this process is not clear (Jones et al., 1999).

In mammals, 5-methylcytosine is limited to CG dinucleotides whereas in plants, methylation can additionally occur at CNG (N = A, T, G, C) and CHH (asymmetric sites; H = A, T, C) sequences. Methylation protects the integrity of the genome by suppressing transposon activity and may also prevent rearrangement in repeat-rich regions (Bender, 2004; Chan et al., 2005; Henderson and Jacobsen, 2007; Martienssen et al., 2005). It also is critically important for gene regulation during development. Null mutations in mouse methyltransferase genes result in embryonic lethality (Goll and Bestor, 2005), while in Arabidopsis thaliana mutations in methyltransferases involved in CG maintenance methylation (met1) or non-CG methylation (ddc triple mutants, drm1/2 and cmt3) lead to severe developmental abnormalities (Chan et al., 2006; Mathieu et al., 2007; Saze et al.,
Alterations in DNA methylation can have detrimental outcomes, such as oncogenesis, in adult organisms as well (Jones and Baylin, 2002; Plass, 2002).

The geminiviruses are a diverse family of single-stranded DNA pathogens that infect a wide variety of plant species and are responsible for significant crop losses (Hanley-Bowdoin et al., 2004; Rojas et al., 2005). These viruses have small (2.5-3.0 kb), circular, monopartite or bipartite genomes that specify four to seven proteins. Geminiviruses do not encode polymerases and are therefore dependent on host machinery for replication and transcription, which occur on viral chromatin templates that consist of double-stranded DNA replicative intermediates associated with cellular histones (Pilartz and Jeske, 2003). Thus, they are attractive models for fundamental host processes, including the epigenetic regulation of replication and gene expression.

It is well established that plants employ PTGS as an adaptive defense against RNA viruses and that, as a countermeasure, viruses encode an array of silencing suppressor proteins (Ding and Voinnet, 2007; Li and Ding, 2006; Roth et al., 2004; Voinnet, 2005a). DNA virus transcripts are also vulnerable to cytoplasmic PTGS, and several proteins produced by geminiviruses and their satellites are known to act as silencing suppressors (Bisaro, 2006; Vanitharani et al., 2005). In addition, there is considerable evidence that plants methylate geminivirus chromatin as an epigenetic defense. Early studies indicated that in vitro methylation of geminivirus DNA greatly impaired replication and transcription in protoplasts (Brough et al., 1992; Ermak et al., 1993). More recently, we have shown that methylation-deficient Arabidopsis mutants are hypersusceptible to geminiviruses and that RdDM pathway components (e.g.
ARGONAUTE 4) are necessary for host recovery from infection. Geminivirus DNA and associated histones are methylated in infected plants, and viral DNA methylation is reduced in mutants that display enhanced disease. By contrast, the small amount of viral DNA present in recovered tissue is hypermethylated (Raja et al., 2008). Given that methylation, and likely TGS, acts as a defense against DNA viruses, we hypothesized that geminiviruses produce proteins capable of interfering with these processes. In this study, we examine the role of AL2 and L2 proteins in this regard. The 15 kDa AL2, also known as AC2, C2, or TrAP (transcriptional activator protein), is encoded by members of the genus Begomovirus, including Tomato golden mosaic virus (TGMV) and Cabbage leaf curl virus (CaLCuV). AL2 is a transcription factor required for the expression of late viral genes (Sunter and Bisaro, 1992). By contrast, the related L2 protein found in the Curtovirus genus, including Beet curly top virus (BCTV), is not a transcription factor (Hormuzdi and Bisaro, 1993). AL2 can suppress PTGS by a mechanism that depends on its ability to activate transcription (transcription-dependent suppression) (Trinks et al., 2005). In addition, both AL2 and L2 can inactivate adenosine kinase (ADK) (Wang et al., 2003), which is required for the efficient production of S-adenosyl methionine (SAM), an essential methyltransferase co-factor (Moffatt et al., 2002). We have demonstrated that AL2 and L2 can inhibit PTGS by a mechanism that involves ADK inactivation (transcription-independent suppression) (Wang et al., 2005; Yang et al., 2007). Here we present evidence that these proteins use a similar mechanism to reverse TGS, and that expression of AL2 and L2 in transgenic plants causes genome-wide reductions in cytosine methylation.
2.2  Materials and Methods

2.2.1 Primers

Primer sequences used for cloning, genotyping, and RT-PCR are listed in Figure 2.9.

2.2.2 Silenced GFP-transgenic *N. benthamiana*.

*N. benthamiana* line 16C containing an active 35S-GFP transgene was inoculated with TRV::35S that was previously shown to cause heritable TGS of the transgene (Jones et al., 2001; Ruiz et al., 1998). After 3-4 weeks, flowers were observed under UV light, and silenced (red) flowers were tagged and seeds harvested. Resulting plants were allowed to self-fertilize to generate 16-TGS lines, and heritable silencing was confirmed in T2 generation seedlings under UV light and by nuclear run-on analysis as previously described (Sunter and Bisaro, 1992). Line 16C and TRV::35S were kindly provided by David Baulcombe.

2.2.3 Recombinant Potato Virus X (PVX) and Tobacco Rattle Virus (TRV) vectors

The binary PVX vector pgR106 (Lu et al., 2003), and the TRV binary vectors pTV00 (TRV RNA 2) and pBINTRAA6 (TRV RNA 1) (Ratcliff et al., 2001) were gifts of David Baulcombe. Recombinant PVX was constructed using AL2, AL2_{114}, AL2-C33A,
and L2 sequences from previously described constructs (Wang et al., 2005; Yang et al., 2007). All genes were first cloned into pUC19, re-sequenced, and added to the pgR106 vector by standard cloning procedures as Ascl-Sall or a Clal-Sall fragments. For TRV vectors, MET1, SAHH, and ADK sequences from cloned N. benthamiana cDNAs were used (Wang et al., 2005) (and unpublished). Portions of these genes (~500 nt SAHH, ~600 nt ADK, ~750 nt MET1) were cloned into pTV00 as Clal-Sall fragments. The resulting PVX and TRV vectors were transformed into the Agrobacterium tumefaciens strain GV3101 and glycerol stocks stored at -80°C prior to use.

2.2.4 Infection of 16-TGS N. benthamiana with recombinant viruses

Two to three weeks post germination, 16-TGS plants were observed under UV light and any showing GFP expression were eliminated. Infiltration of plants with A. tumefaciens GV3101 harboring the PVX vectors was carried out as previously described (Wang et al., 2005). Successful infection was indicated by mosaic PVX symptoms after 7 days. A similar procedure was used for pTV00-based vectors, except that a culture containing pBINTRA6 was added at a 2:1 ratio prior to infiltration. Because TRV normally produces asymptomatic infections, VIGS was confirmed in parallel using pTV09, which targets the sulfur allele of magnesium chelatase and causes photobleaching in infected plants (Kjemtrup et al., 1998). After 4-6 weeks, infected plants were photographed under UV light using a Nikon Coolpix 990 handheld digital camera, and tissue samples taken for RNA analysis.
2.2.5 Transgenic *Arabidopsis* studies

A dexamethasone (dex) induction system was chosen to make AL2, AL2\textsubscript{1-114}, L2, and dsADK transgenic lines (Aoyama and Chua, 1997; McNellis et al., 1998). The TGMV AL2, AL2\textsubscript{1-114}, and BCTV L2 genes were cloned into pTA7001, containing a dex-inducible promoter, as SpeI-XhoI fragments. The dsADK construct was generated by insertion of an inverted repeat sequence corresponding to ~600 nt of *Arabidopsis ADK2* cDNA (Wang et al., 2003). The TA7001-AL2, AL2\textsubscript{1-114}, L2, and dsADK constructs were transformed into the *Agrobacterium* strain GV3101, and then into the *Colombia* ecotype of *Arabidopsis thaliana* using the floral dip procedure (Bent, 2006). T1 seeds were selected on MS medium containing B vitamins and 30 µg/ml hygromycin. Plants with well-developed cotyledons and roots were transplanted to soil and genotyped with primers specific for the transgene and dex promoter cassette using the Extract-N-Amp PCR kit (Sigma). T2 seeds were harvested from positive lines, selected again on media containing hygromycin, and the resulting plants used for experiments.

Transgenic plants were grown until bolting (3-5 weeks). Dexamethasone stock solution (20 mM in 100% ethanol) was diluted 1:1000 into sterile water containing 0.05% Silwet L-77 (Vac-In-Stuff, Lehle Seeds). The diluted dex (or water) was sprayed onto plants with a Paasche VLSTPRO double-action airbrush attached to a Husky (Home Depot, Inc.) 4-gallon air compressor set to 40 psi. After spraying, plants were laid on damp paper towels under a dome for 12-24 hour, then returned to the growth chamber for 24-48 hours. Plants were re-sprayed every 48-72 hrs for 1-2 weeks, then samples taken.
for RNA analysis (see below). ADK activity present in dsADK transgenic lines was measured as previously described (Wang et al., 2003).

2.2.6 RNA extraction and analysis

RNA extraction, Northern blot, and RT-PCR procedures have been described (Wang et al., 2005). Briefly, 1 ml Trizol reagent (Invitrogen) was used to extract RNA from 0.1 g of plant tissue. Northern blots were performed with 5-10 µg of RNA. Antisense riboprobes were synthesized with [α-32P]UTP and the Maxiscript T7 kit, and DNA probes with [α-32P]dCTP and the Strip-EZ DNA kit (Applied Biosystems). RT-PCR was carried out using the Superscript III One-Step RT-PCR kit (Invitrogen) with 1 µg of RNA.

Appropriate enzymes were selected based in target AtSN1 and Athila sequences

2.2.7 Methylation-sensitive PCR

using REBASE (New England Biolabs), a database containing methylation sensitivity data for restriction endonucleases. One hundred ng of genomic DNA from either dex- or water-treated transgenic Arabidopsis plants was incubated with 2 units of methylation-sensitive restriction enzyme in 20 µl reactions for at least 14 hr. Enzymes were heat-inactivated, then 20 ng of the cleaved DNA was loaded into PCR reactions containing primers for the target loci. Primers specific for Arabidopsis 18S rDNA sequence served as controls.
2.2.8 Bisulfite sequencing

Bisulfite sequencing of \textit{AtSN1} was carried out as previously described (Frommer et al., 1992; Raja et al., 2008) using PCR primers specific for \textit{AtSN1} (Zilberman et al., 2003).

2.2.9 Methylation-sensitive extension assay

The extension assay was performed essentially as described (Boyko et al., 2007; Pogribny et al., 1999). \textit{Arabidopsis} genomic DNA (1 \textmu g) was digested for 16 hr with a 10-fold excess of \textit{MspI}. An additional 1 \textmu g DNA aliquot from the mock samples was incubated without restriction enzyme and served as a background control. Single-nucleotide extension reactions were carried out using 500 ng DNA, 1 x PCR Buffer (Invitrogen), 1.0 mM MgCl$_2$, 0.25 units of Taq DNA polymerase (New England Biolabs, Beverly, MA), 0.5 \textmu l [$\alpha$-\textsuperscript{32}P]dCTP (800 Ci/mmol) (Perkin-Elmer) for 1 hour in 25 \textmu l reactions. 10 \textmu l was loaded onto each of two DE-81 filters (Whatman) and washed as described. Radioactivity was determined by scintillation counting.
2.3 Results

2.3.1 AL2 and L2 reverse TGS of a GFP transgene in a transcription-independent manner.

To investigate the effects of AL2 and L2 on TGS, we created *Nicotiana benthamiana* lines containing a transcriptionally silenced GFP transgene. 16c plants, which contain an active GFP transgene driven by the 35S promoter, were inoculated with a *Tobacco rattle virus* (TRV) vector expressing a portion of the 35S promoter sequence to induce TGS (Jones et al., 2001; Ruiz et al., 1998). Plants were observed under UV light and seed was collected from red flowers, where GFP expression was silenced. The resulting silenced progeny were self-fertilized to establish silenced (16-TGS) lines. Nuclear run-on analysis confirmed that the heritable silencing in 16-TGS plants was transcriptional in nature (Figure 2.1 D).

TGS reversal assays were carried out using *Potato virus X* (PVX) vectors to express TGMV AL2 and BCTV L2 proteins (Voinnet et al., 1999). Silenced 16-TGS plants were inoculated with PVX (empty vector control), PVX::AL2, PVX::AL2$_{1-114}$, PVX::AL2 C33A, and PVX::L2. AL2$_{1-114}$ lacks the C-terminal 15 amino acids that constitute the transcription activation domain, and the AL2 C33A mutant protein has reduced transcription activation activity (about one-third of wild-type) (Hartitz et al., 1999; Yang et al., 2007). PVX infection had no impact on GFP silencing. However, the
geminivirus proteins intensified PVX symptoms (not shown), and GFP expression was evident by UV inspection in all 16-TGS plants inoculated with all PVX::AL2/L2 recombinant viruses. Yellow-green GFP fluorescence was especially apparent in leaf petioles and upper leaves where PVX symptoms were most pronounced (Figure 2.1 A). The presence of GFP mRNA was confirmed by Northern blot analysis (Figure 2.1 C).

TRV vectors were employed to knock-down the expression of selected cellular genes by virus-induced gene silencing (VIGS) (Ratcliff et al., 2001). The 16-TGS plants were inoculated with TRV (empty vector control), TRV::MET1 (positive control), TRV::SAHH, and TRV::ADK. The 500 to 750 nt targeting sequences used in these experiments were obtained from cloned *N. benthamiana* cDNAs (Wang et al., 2005) (and unpublished). MET1 is a CG-specific methyltransferase required for TGS (Jones et al., 2001). SAHH refers to S-adenosyl homocysteine hydrolase, an enzyme essential to the methyl cycle that generates SAM and which also is required for TGS (Rocha et al., 2005). The ADK construct targeted the methyl cycle associated enzyme that is inhibited by AL2 and L2 (Moffatt et al., 2002; Wang et al., 2003) (A diagram of the methyl cycle is presented in Figure 1.5). As illustrated in Figures 2.1 B and 2.1 C, all TRV treatments, except the empty vector, were able to restore GFP expression. In addition, the targeting sequences also enhanced TRV symptoms, which normally are very mild or absent (not shown). However, unlike the viral proteins which reversed silencing to roughly equivalent extents, clear differences in effectiveness were noted. MET1 targeting resulted in the most robust GFP expression, followed in order by SAHH and ADK. We concluded that AL2 and L2 can reverse TGS of a GFP transgene by a
mechanism that does not depend on transcriptional activation, and which correlates with methyl cycle inhibition.

2.3.2 AL2 and L2 cause ectopic expression of TGS-silenced *Arabidopsis* loci by transcription-dependent and –independent mechanisms.

To confirm and further evaluate TGS reversal activity, we constructed transgenic *Arabidopsis* lines expressing AL2, AL21-114, L2, or GFP (negative control) from a dexamethasone (dex)-inducible promoter. An inducible expression system was chosen because previous studies showed that expression of full-length AL2 is not compatible with development (Sunter et al., 2001). Transgenes were confirmed by genomic PCR, and expression levels were verified by Northern blot analysis or semi-quantitative (sq) RT-PCR (not shown). At least two independent transgenic lines expressing AL2, AL21-114, or L2 mRNAs following dex treatment, but not after water (mock) treatment, were selected for this study. In each experiment, extracts from individual plants were re-tested for transgene expression by Northern blot or sqRT-PCR. Those showing high levels of transgene mRNAs following dex treatment were again used for sqRT-PCR with primers to amplify transcripts from selected endogenous loci known to be silenced by methylation, or from control genes (Tran et al., 2005; Zhang et al., 2006).

Following dex treatment of AL2 plants, ectopic expression was observed from all silenced loci tested, including a putative *F-box* family protein (At2g17690), the retrotransposons *AtSN1* (SINE element) and *Athila* (LTR element), and a *CACTA*-like DNA transposon (At2g04770) (Figure 2.2). All but one of these (*CACTA*-like) were also
over-expressed in dex-treated AL2₁₋₁₁₄ and L2 plants. Plants expressing GFP (negative control) showed no increase in transcription of any of the tested sequences (not shown).

It is not yet clear why the expression of CACTA-like was enhanced only by AL2. However, it is consistent with the idea that this protein can suppress silencing by transcription-dependent and transcription-independent means, whereas AL2₁₋₁₁₄ and L2 are limited to the latter mechanism. Because the transcription-independent mechanism involves inhibition of cellular ADK activity, we next examined the consequences of dex-induced expression of an inverted repeat transgene corresponding to Arabidopsis ADK2 (dsADK). Due to the high level of similarity between the two Arabidopsis ADK genes (88% identity at the nucleotide level), we expected dsRNA produced from the dsADK transgene to target both mRNAs by RNAi. Plants from two independent, transgenic lines with reduced ADK activity following dex induction were selected (Fig. 3A), and the expression of silenced loci was examined by sqRT-PCR. Remarkably, over-expression of Athila and AtSN1, but not CACTA-like, was evident in extracts from dex-induced dsADK plants (Figure 2.3 B). We concluded that transgenic expression of AL2, AL2₁₋₁₁₄, L2, and dsADK can reverse TGS of certain endogenous loci silenced by methylation and that, consistent with the transcription-independent suppression mechanism, dsADK treatment phenocopies AL2₁₋₁₁₄ and L2. However, TGS suppression of the CACTA-like sequence appears to require the transcription-dependent mechanism of AL2.
2.3.3 Transgenic expression of AL2 reduces cytosine methylation at previously silenced loci.

To determine whether the ectopic expression observed following AL2 induction was associated with reduced cytosine methylation, we first analyzed *Athila* and *AtSN1* genomic sequences using methylation-sensitive PCR. This method involves digestion of genomic DNA with restriction endonucleases that are unable to cleave methylated target sequences, followed by PCR with locus-specific primers that flank a target site. Destruction of template due to reduced methylation is expected to result in a smaller amount of PCR product.

We tested several endonucleases which are blocked by methylation of the underlined cytosine residues, including *AlwNI* (CAGNNNCTG), *BmrI* (ACTGGG), *BspCNI* (CTCAG), *EcoO109I* (A/GGGNCCC/T), and *HpyCH4V* (TGÇA). We observed that 2 of 6 sites tested in *Athila*, and 3 of 12 sites in *AtSN1*, showed obvious decreases in PCR product in extracts prepared from AL2 plants treated with dex compared to water-treated plants (Figure 2.4). These results strongly suggested that the presence of AL2 resulted in partial demethylation of cytosine residues in some of the sites examined.

To investigate methylation status at higher resolution, bisulfite sequencing of a 156 bp fragment of *AtSN1* was performed. Within this sequence, there are 4 CG, 7 CNG, and 33 CHH sites. DNA obtained from dex- or water-treated AL2 plants was treated with bisulfite reagent to convert unmethylated cytosines to uracil, followed by PCR to amplify the *AtSN1* locus. PCR products were cloned and the sequences of six clones were compiled for each treatment. We found that the presence of AL2 resulted in a 14%
decrease in the total number of cytosine residues methylated, with most reductions occurring at non-CG sites (~28% CNG; 13% CHH) (Figure 2.5). (Primary sequence data is presented in Figure 2.7)

2.3.4 Transgenic expression of AL2 or L2 causes genome-wide reductions in cytosine methylation

To gain a more global view of the effects of AL2 or L2 on the methylation status of genomic DNA, we adopted a methylation-sensitive extension assay (Boyko et al., 2007; Pogribny et al., 1999). These experiments took advantage of the fact that methylation of the external cytosine in the target site of \( MspI \) (\( \text{C}^{\downarrow}\text{CGG} \)) prevents cleavage. (Methylation of the internal cytosine has no effect on \( MspI \) activity). Following incubation of genomic DNA with \( MspI \), a single nucleotide extension assay was performed using \([\alpha^{32}\text{P}]-\text{dCTP} \) and Taq DNA polymerase, which lacks a 3' → 5' exonuclease activity. Assuming complete extension, the extent of nucleotide incorporation depends on the number of \( MspI \) sites cleaved which in turn depends on the degree of cytosine methylation, in this case at CNG sites within the \( MspI \) recognition sequence.

We first confirmed that treatment of wild-type plants with dex, which is delivered in a solution containing 0.05% Silwet L77 to facilitate cuticle penetration, had no significant impact on methylation compared to water treatment (Figure 2.8 B). We also determined that extension reactions were ~90% complete by 15 minutes, reached maximum levels at 45 minutes, and remained at this plateau for at least 120 minutes.
As an additional control, we also demonstrated that pUC plasmid DNA added to excess genomic DNA extract was cleaved to completion by *MspI* under our experimental conditions (not shown).

Methylation extension assays were then performed for 60 minutes using individual DNA extracts from three dex-treated plants from each of two independent AL2 or L2 transgenic lines. Extracts from water-treated AL2 or L2 plants were used as controls. The results of this analysis indicated that expression of AL2 resulted in a 1.5 to 2.3-fold increase, and expression of L2 in a 2.1 to 2.6-fold increase, in incorporation of labeled cytosine (Figure 2.6). Students' *t*-test confirmed that the differences were significant at the 95% to 99% confidence interval. Thus expression of AL2 and L2 resulted in increased *MspI* cleavage, which reflects a corresponding global decrease in methylation levels at the CNG sites queried.

### 2.4 Discussion

Previous work has demonstrated that plants employ RNA-directed methylation of geminivirus DNA and associated histone proteins as an antiviral defense mechanism (Raja et al., 2008). Methylation likely leads to epigenetic transcriptional silencing of geminivirus chromatin. Here, using complementary approaches that exploit the advantages of *N. benthamiana* and *Arabidopsis* experimental systems, we present evidence that geminivirus AL2 and L2 proteins are able to counter this defense. Both proteins were shown to reverse TGS of a GFP transgene following expression from a PVX vector in *N. benthamiana*. Following dex-induced expression of AL2 and L2
transgenes in *Arabidopsis*, both proteins could reverse TGS of selected loci known to be silenced by methylation. To our knowledge, this is the first report that viral proteins can reverse TGS. However, given the similarities between small RNA-directed pathways in plants, the functional redundancy between pathway components, and the variety of viral silencing suppressors that inhibit various pathway enzymes or bind dsRNA, we anticipate that other DNA and RNA virus proteins will also prove capable of inhibiting TGS.

Earlier studies suggested that AL2 can suppress PTGS by at least two distinct mechanisms. A transcription-dependent mechanism is believed to involve the activation of host genes (e.g. *Werner exonuclease-like 1*; *WEL1*) that function as endogenous negative regulators of RNA silencing pathways (Trinks et al., 2005; van Wezel et al., 2003). A second, transcription-independent mechanism is shared with L2 and mutant AL2 proteins that are defective for transcription activation (e.g. AL2<sub>1-114</sub> and AL2<sub>C33A</sub>). In this case PTGS suppression results from interaction with and inhibition of ADK, which is required for optimal transmethylation activity (Wang et al., 2005; Wang et al., 2003; Yang et al., 2007). The studies presented here both support and extend the scope of the transcription-independent mechanism by showing that AL2, AL2<sub>1-114</sub>, AL2<sub>C33A</sub>, and L2, as well as VIGS targeting of ADK and the essential methyl cycle enzyme SAHH, can reverse TGS directed against a GFP transgene in *N. benthamiana*. That AL2, AL2<sub>1-114</sub>, L2, and dsADK transgenes cause ectopic expression of *Arabidopsis AtSN1*, *Athila*, and *F box* loci that are transcriptionally silenced by methylation provides powerful additional support. An interesting exception was the *CACTA-like* transposon, which is apparently refractory to the transcription-independent mechanism. The reasons
for this are presently unclear. However, it is known that the $AtSN1$, $Athila$, and $F$ box loci are ectopically expressed in mutant backgrounds that primarily impact non-CG methylation (e.g. $ddc$) (Tran et al., 2005; Zhang et al., 2006). By contrast, $CACTA$-like expression is observed in a $met1$ mutant background, suggesting that CG methylation is important for silencing at this locus (Zhang et al., 2006). Perhaps CG methylation patterns are more robustly maintained than non-CG methylation, and thus less likely to be degraded by methyl cycle inhibition. Studies to determine how methylation in different sequence contexts is affected by AL2 and L2 are in progress. In this regard, it has been noted that a number of transposons are reactivated by mutations that cause demethylation, and that for some (including CACTA), methylation in different contexts may function in an additive manner to bolster silencing (Kato et al., 2003; Miura et al., 2001; Singer et al., 2001). It will be interesting to see whether transgenic expression of AL2 and L2 causes differential reactivation of transposon classes and if so, whether this also occurs in infected cells. Our work raises the possibility that host genome instability caused by transposon reactivation is a component of geminivirus pathogenesis.

Using restriction-sensitive PCR and bisulfite sequencing, we demonstrated that increased transcription of $Athila$ and $AtSN1$ following dex-induced expression of an AL2 transgene is accompanied by reduced methylation of these loci. However, because methylation inhibition via ADK inactivation is not expected to be locus-specific, we predicted that AL2 and L2 could affect global methylation patterns. Using a methylation-sensitive extension assay capable of assessing the CNG methylation status of $MspI$ sites genome-wide, we were able to confirm that reduced methylation caused by the viral
proteins is indeed non-specific in nature. Thus AL2 and L2 could prove to be useful tools for attenuating global cytosine methylation as well as other types of cellular transmethylation events that require SAM as a co-factor, such as histone methylation.

In summary, we conclude that geminivirus AL2 and L2 proteins can suppress TGS by interfering with methylation, which is accomplished by inhibition of ADK. TGS suppression activities would be expected to occur in DNA viruses that are subject to genome methylation, and their existence reinforces the importance of this process as an epigenetic defense in plants. It is possible that mammalian DNA viruses are confronted with similar defenses, and certainly there are many (e.g., herpesviruses) that have latency pathways characterized by hypermethylation of the viral genome. Whether mammalian viruses produce proteins capable of directly suppressing methylation and/or reversing TGS remains to be seen.
Figure 2.1 AL2 and L2 reverse TGS of a GFP transgene.

(A) Transgenic *N. benthamiana* plants containing a transcriptionally silenced 35S-GFP transgene (line 16-TGS) were inoculated with a PVX vector, or PVX expressing the indicated viral proteins. Plants were photographed under UV light two weeks post-inoculation. Uniform dark red color resulting from chlorophyll autofluorescence in the absence of GFP is evident in the PVX-inoculated plant. Release of TGS is indicated by the presence of GFP (yellow-green color) in veins and mesophyll of upper leaves following inoculation with PVX::AL2, AL21-114, AL2-C33A, and L2. Results are representative of at least three independent trials with 4-8 plants per treatment.

(B) As in A, except 16-TGS plants were inoculated with a TRV VIGS vector or TRV expressing sequences derived from the indicated endogenous genes in order to reduce their expression.

(C) Northern blot of RNA from leaves of inoculated 16-TGS plants. The $^{32}$P-labeled probe was specific for GFP mRNA. The 18S rRNA loading controls were visualized by staining the gel with ethidium bromide.

(D) Nuclear run-on analysis indicating GFP transcription in line 16C and transcriptional silencing in derivative line 16-TGS. Rubisco served as a control.
Figure 2.1 AL2 and L2 reverse TGS of a GFP transgene.
Figure 2.2 AL2\textsubscript{1-14}, and L2 cause over-expression of methylated, TGS-silenced loci. *Arabidopsis* plants containing the indicated transgenes under control of a dex-inducible promoter were treated with dex or water, and expression from the endogenous silenced loci was evaluated by sqRT-PCR. Actin served as a control. The data are representative of results obtained in at least two experiments using two independent lines for each transgene.
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**Figure 2.2** AL2<sub>1-114</sub>, and L2 cause over-expression of methylated, TGS-silenced loci.
Figure 2.3 ADK dsRNA causes ectopic expression of TGS-silenced loci.

(A) Arabidopsis plants containing a dex inducible transgene designed to express ADK dsRNA (dsADK) were treated with dex or water, and ADK activity was evaluated. The histogram shows relative ADK activity in extracts of plants from two independent lines (dsADK-L4 and dsADK-L5) following dex or water (mock) treatment. Mock extracts were obtained from a water-treated, dsADK-L4 plant.

(B) Expression of the indicated endogenous loci in extracts from dsADK plants treated with dex or water was assessed by sqRT-PCR. 18S rRNA served as control. Lane 1, mock; lane 2, dsADK-L4; lane 3, dsADK-L5.
Figure 2.3 ADK dsRNA causes ectopic expression of TGS-silenced loci.
Figure 2.4 Restriction-sensitive PCR shows cytosine methylation in the *Athila* and *AtSN1* loci following expression of AL2.

*Arabidopsis* plants containing a dex-inducible AL2 transgene (one- to two independent lines) were treated with dex or water. DNA extracts were incubated with the indicated, methylation sensitive restriction enzymes prior to genomic PCR with primers flanking the restriction site. Destruction of template due to reduced methylation is expected to result in a smaller amount of PCR product. 18S rDNA served as control.
Figure 2.4 Restriction-sensitive PCR shows cytosine methylation in the *Athila* and *AtSN1* loci following expression of AL2.
**Figure 2.5** Bisulfite sequencing reveals reduced cytosine methylation in the *AtSN1* locus following expression of AL2.

DNA was obtained from transgenic dex-AL2 *Arabidopsis* plants after spraying with dex or water. Following bisulfite treatment, the *AtSN1* locus was amplified by PCR and cloned. Six clones each were sequenced from dex- or water-treated plants.

**(A)** Summary of bisulfite sequencing data. Red represents CG sites, green CNG sites, and blue CHH sites. Height of bars is proportional to the number of clones with a methylated cytosine, ranging from 0 to 6. The 156 nt sequence is divided into three parts, with data from water (mock) treatment shown above data from dex-treatment.

**(B)** The histograms represent the proportion of cytosine residues methylated in different sequence contexts.
**Figure 2.5** Bisulfite sequencing reveals reduced cytosine methylation in the *AtSN1* locus following expression of AL2.
Figure 2.6 Transgenic expression of AL2 and L2 reduces genome-wide cytosine methylation.

The histograms illustrate relative incorporation of dCTP observed in methylation-sensitive extension assays. DNA was obtained from dex- or water treated Arabidopsis plants containing dex-AL2 or dex-L2 transgenes, digested to completion with MspI, and incubated with $^{32}$P-dCTP and Taq polymerase to allow single nucleotide extension (see text). Increased incorporation reflects enhanced MspI cleavage due to reduced methylation. In each case, assays were performed with DNA from three individual plants from each of two independent AL2 (AL2-L6 and AL2-L7) and L2 (L2-L6 and L2-L8) transgenic lines. Mock treatment assays were done using DNA from three to four plants water-treated from the same transgenic lines. Asterisks indicate significant differences between dex- and mock-treated samples at the 95% (*) or 99% (**) confidence level, as determined by Students' $t$-test.

(A) AL2 transgenic lines.

(B) L2 transgenic lines.
**Figure 2.6** Transgenic expression of AL2 and L2 reduces genome-wide cytosine methylation.
Figure 2.7 Bisulfite sequencing reveals reduced cytosine methylation in the AtSN1 locus following expression of AL2.

DNA was obtained from transgenic dex-AL2 Arabidopsis plants after spraying with dex or water. Following bisulfite treatment, the AtSN1 locus was amplified by PCR and cloned. Primers are listed in Figure 2.9. Six clones each were sequenced from dex- or water-treated plants.

(A) Primary sequencing data from mock (water)-treated AL2 plants. In the reference sequence (Ref), red indicates CG sites, violet CNG sites, and gray CHH sites. For the experimental sequences, yellow represents methylated cytosines and green unmethylated, converted (T) sites.

(B) As in (A), except experimental sequences are from dex-treated AL2 plants.
Figure 2.7 Bisulfite sequencing reveals reduced cytosine methylation in the AtSN1 locus following expression of AL2.

Continued on next page
Figure 2.8 Control experiments for the methylation-sensitive extension assay.

(A) Extension reaction time course. *Arabidopsis* genomic DNA (5 µg) was digested for 16 hr with a 10-fold excess of *MspI*. Single-nucleotide extension reactions were carried out using 500 ng DNA, 1 x PCR Buffer (Invitrogen), 1.0 mM MgCl$_2$, 0.25 units of Taq DNA polymerase (New England Biolabs, Beverly, MA), 0.5 µl [$^{32}$P]dCTP (800 Ci/mmol) (Perkin-Elmer) for the indicated times in 25 µl reactions. 10 µl was loaded onto each of two DE-81 filters (Whatman) and washed. Radioactivity was determined by scintillation counting.

(B) Effect of dex versus water treatment on incorporation. The histograms illustrate relative incorporation of dCTP observed. DNA was obtained from dex- or water treated, wild-type *Arabidopsis* plants (Col-0), digested to completion with *MspI*, and incubated with $^{32}$P-dCTP and Taq polymerase to allow single nucleotide extension. Experiments were performed with DNA from four individual plants for each treatment.
Figure 2.8 Control experiments for the methylation-sensitive extension assay.
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<sup>a</sup>GVG primers that amplify a region of the dex inducible promoter were used for genotyping in transgenic plants.

<sup>b</sup>AtSN1 primers were used for RT-PCR and bisulfite sequencing

**Figure 2.9** Primers used for RT-PCR, genotyping, bisulfite sequencing.
CHAPTER 3

ANALYSIS OF ALTERNATIVE GEMINIVIRUS AL2 AND L2 SILENCING SUPPRESSION ACTIVITIES SUGGESTS MULTIPLE PATHWAYS FOR MAINTENANCE OF POST-TRANSCRIPTIONAL GENE SILENCING

The following chapter will be submitted for publication as follows: Buchmann, R.C., Mubeen, M. and Bisaro, D.M. Analysis of alternative geminivirus AL2 and L2 silencing suppression activities suggests multiple pathways for maintenance of post-transcriptional gene silencing. Figure 3.2 was done in collaboration with Mohammed Mubeen.

3.1 Introduction

Viruses belonging to the Geminiviridae package small, circular single-stranded DNA (ssDNA) genomes in unique double-icosahedral particles. The ssDNA genome, which may be monopartite or bipartite, is replicated by rolling circle and recombination-mediated mechanisms that utilize double-stranded DNA (dsDNA) intermediates that associate with cellular histones to form minichromosomes. Replication and transcription from viral templates is accomplished by host polymerases and accessory proteins. Viral
gene products are typically multifunctional proteins that collectively direct host machinery to viral templates, initiate specific steps in replication and/or transcription, enable virus spread within and between hosts, provide a cellular environment favorable to replication, and suppress host defenses (Gutierrez, 1999; Hanley-Bowdoin et al., 1999; Hanley-Bowdoin et al., 2004; Rojas et al., 2005).

RNA silencing is a general term that refers to a set of mechanistically related and evolutionarily conserved processes often called post-transcriptional gene silencing (PTGS) in plants and RNA interference (RNAi) in animals. In plants, PTGS usually leads to the cytoplasmic degradation of mRNAs produced from transgenes and certain endogenous mRNAs. An antiviral role is also well-established, and the importance of PTGS as a defense is clear from the fact that virtually all plant viruses encode proteins that act as silencing suppressors (Ding and Voinnet, 2007; Li and Ding, 2006; Roth et al., 2004; Voinnet, 2005a). These counterdefensive proteins are essential pathogenicity factors that act by a variety of mechanisms to block different aspects of silencing. A remarkable feature of silencing that greatly enhances its defensive value is its ability to spread cell-to-cell and systemically throughout the plant. However, the molecular basis for systemic spread, and the mechanisms by which some viral suppressors are able to prevent it, remain to be elucidated. Likewise, some viral suppressors are known to reverse established silencing, suggesting that they target silencing maintenance pathways about which little is known.

An example of a geminivirus silencing suppressor is AL2 encoded by members of the genus Begomovirus, which includes Tomato golden mosaic virus (TGMV) and
Cabbage leaf curl virus (CaLCuV). The 15-kDa AL2 (also known as AC2, C2, or TrAP, transcriptional activator protein) was originally characterized as a transcription factor required for the expression of virus late genes (Sunter and Bisaro, 1992; Sunter and Bisaro, 2003). In contrast, the related L2 protein found in the Curtovirus genus, for example Beet curly top virus (BCTV), is not required for late viral gene expression and lacks transcription activation activity (Hormuzdi and Bisaro, 1995; Yang et al., 2007). However, AL2 and L2 share pathogenicity functions. AL2 has been shown to suppress PTGS by multiple mechanisms (Bisaro, 2006). One involves AL2-mediated activation of host genes that may act as endogenous negative regulators of RNA silencing (transcription-dependent suppression) (Trinks et al., 2005; van Wezel et al., 2003). The other is based on the ability of both AL2 and L2 to interact with and inactivate adenosine kinase (ADK), a methyl cycle-associated enzyme required for the efficient production of S-adenosyl methionine (SAM), an essential methyltransferase cofactor (Moffatt et al., 2002; Wang et al., 2003). We have demonstrated that AL2 and L2 can suppress local silencing (PTGS) by a mechanism that likely depends on inhibition of the methyl cycle through ADK inactivation (transcription-independent suppression) (Wang et al., 2005; Yang et al., 2007). One goal of the present study was to obtain further support for this mechanism by asking whether S-adenosyl homocysteine hydrolase (SAHH), an essential methyl cycle enzyme, is required for local silencing.

More recently, we demonstrated that small RNA-directed methylation, and likely transcriptional gene silencing (TGS), acts as an epigenetic defense against geminiviruses, and that AL2 and L2 can suppress TGS by both transcription-dependent and –
independent mechanisms (Buchmann et al., 2008; Raja et al., 2008). However, the role of these two mechanisms in suppression of different aspects of PTGS remained to be described. Thus a second goal was to determine whether, and in general terms how, AL2 and L2 inhibit systemic spread of silencing or cause the reversal of PTGS.

3.2 Results

3.2.1 SAHH is required for local PTGS

We previously demonstrated that TGMV AL2, AL2 lacking the transcription activation domain (AL2\textsubscript{1-114}), BCTV L2, and treatments that inhibit ADK activity, including use of an RNAi construct (dsADK), could suppress local silencing directed against GFP mRNA (Wang et al., 2005; Yang et al., 2007). This strongly suggested that inhibition of the methyl cycle, through AL2 and L2-mediated inactivation of ADK, was responsible for transcription-independent silencing suppression observed in this assay. However, because ADK has many cellular functions, we investigated the importance of SAHH, an essential methyl cycle enzyme, for local silencing.

A two-component silencing assay in *Nicotiana benthamiana* line 16c plants, which contain and actively express a 35S-GFP transgene, was employed (Ruiz et al., 1998). Leaves were co-infiltrated with *Agrobacterium tumefaciens* cultures harboring a Ti plasmid expressing GFP from the 35S promoter as a silencing inducer, along with cultures containing test or control constructs (Guo and Ding, 2002; Johansen and Carrington, 2001; Voinnet et al., 2000). Procedures for expression plasmid construction,
infiltration, visualization of GFP under UV light, and Northern blot analysis of GFP mRNA levels have been described (Wang et al., 2005).

β-glucuronidase (GUS) served as a negative control, and tombusvirus p19 as a positive control. The p19 protein is a very strong suppressor for transgene-induced local silencing (Silhavy et al., 2002), and AL2 was included as another positive control. The test construct was designed to reduce SAHH mRNA levels by RNAi, and expressed an inverted repeat sequence corresponding to *N. benthamiana* SAHH (dsSAHH). To create this plasmid, the Superscript One-Step RT-PCR kit (Ambion) was used to clone a fragment of SAHH mRNA from total *N. benthamiana* cellular RNA using primers derived from exon 3 of the *N. tabacum* sequence (5' primer: TTGCCGGTTATGGAGATGTCGGAA; 3' primer: AAGGACCTTCAACTGGTAACGCTGA). A 419-bp fragment of the SAHH cDNA was inserted into a Ti plasmid-based expression vector in opposite orientations as previously described (Wang et al., 2005). The efficacy of dsSAHH was confirmed by semi-quantitative RT-PCR following infiltration of *N. benthamiana* leaves. As shown in Fig. 1C, SAHH mRNA levels were substantially reduced by the dsSAHH construct.

Following co-infiltration of leaves with cultures delivering GFP and the GUS negative control, weak fluorescence and a red ring surrounding the infiltration zone, due to reduced GFP expression and red chlorophyll autofluorescence under UV light, was observed. By contrast, we noted strong suppression by p19 and somewhat weaker suppression by AL2, as apparent from robust yellow-green GFP fluorescence and absence of the red ring. The presence of the dsSAHH construct also obviously
suppressed GFP-directed silencing (Fig. 3.1A). These results were confirmed by Northern blot. GFP mRNA accumulated to the highest levels in zones infiltrated with p19 and also accumulated to levels substantially higher than the GUS negative control in zones infiltrated with AL2 and dsSAHH (Fig. 3.1B). We concluded that reducing SAHH mRNA levels resulted in suppression of PTGS in this local assay, indicating that local PTGS suppression requires SAHH and an active methyl cycle.

3.2.2 AL2, but not L2, can effectively prevent the spread of systemic silencing.

A similar assay was exploited to examine whether AL2, L2, and methyl cycle inhibition could inhibit the systemic spread of PTGS (Guo and Ding, 2002; Johansen and Carrington, 2001; Voinnet et al., 2000). In this case *N. benthamiana* 16c plants were coinfiltrated with *A. tumefaciens* cultures harboring a Ti plasmid that expresses an inverted repeat RNA (dsGFP) corresponding to the GFP coding region, which acts as a strong inducer of systemic PTGS, and a culture expressing a test gene or control. GUS again served as a negative control and p19 as a positive control because it is also a strong suppressor of systemic silencing (Silhavy et al., 2002). Previously described test constructs included those expressing AL2, L2, AL2<sub>1-114</sub> and AL2-C33A (Wang et al., 2005; Yang et al., 2007). The latter is defective in self-interaction, which is required for optimal transcription activation activity. However, neither this substitution nor the absence of the activation domain affects the AL2:ADK interaction (Yang et al., 2007). In
addition, RNAi constructs expressing inverted repeat RNAs corresponding to *N. benthamiana* ADK (dsADK) and SAHH (dsSAHH) genes were also tested. Following coinfiltration of 16c plants with dsGFP and GUS, systemic silencing of the GFP transgene was visible under UV light after ~10 days on the crown leaves as dark red areas against the salmon color of GFP-expressing tissue, due to chlorophyll autofluorescence (Fig. 3.2). Remarkably, despite its relatively weak activity in local assays, AL2 proved nearly as effective as p19 in inhibiting systemic silencing, with a range of 80-100% unsilenced plants over three independent experiments. This result is consistent with a previous study which showed that AL2 (AC2) protein from *Mungbean yellow mosaic virus* can interfere with the spread of silencing (Trinks et al., 2005). By contrast, AL21-114 and L2, which lack transcription activation activity, did not prevent systemic silencing and were essentially equivalent to the GUS negative control. Both the dsADK and dsSAHH constructs also proved ineffective (Fig. 3.2). These observations indicate that the transcription-independent mechanism is insufficient to prevent the initiation, subsequent spread, or perception of the systemic silencing signal. Underscoring the necessity for transcription activation, AL2-C33A, with about ~30% the activation activity of wild-type AL2, displayed intermediate suppression in this assay (Fig. 3.2). We concluded that AL2, but not L2, can inhibit systemic spread of silencing by a transcription-dependent mechanism.
3.2.3 Plant developmental age determines whether the transcription-dependent or –independent mechanisms can cause reversal of PTGS.

Only a relatively few silencing suppressors, such as HC-Pro and AL2, can reverse established silencing, which suggests they are able to interfere with a maintenance step (Anandalakshmi et al., 1998; Brigneti et al., 1998; Llave et al., 2000; Voinnet et al., 1999).

Analysis of silencing reversal also employed *N. benthamiana* 16c (Brigneti et al., 1998; Voinnet et al., 1999). Plants were infiltrated approximately two weeks post-germination with *A. tumefaciens* delivering dsGFP to trigger systemic silencing, and plants in which GFP silencing spread and become established throughout the plant were used in this study. After infiltration, plants were split into two pools. "Mature" plants were left in small pots (6 cm) and allowed to grow until they began to produce flowers. "Immature" plants were transplanted into larger (16.5 cm) pots, which allowed them to continue vegetative growth. Thus plants of different developmental ages with respect to the vegetative-to-floral transition were tested. Silenced plants were inoculated with recombinant *Potato virus X* (PVX) vectors expressing AL2, AL2_{114}, and L2, constructed as described (Buchmann et al., 2008). PVX (empty vector) served as a negative control, and HC-Pro as positive control. Four weeks post-inoculation, plants were observed under UV light and samples taken for analysis of GFP mRNA.

After two weeks, GFP fluorescence was not reliably observed in previously silenced, intact plants, even with the HC-Pro positive control. We then examined water-mounted tissue sections using a confocal microscope, and under these conditions yellow-
green GFP-expressing cells could easily be seen against a background of red cells in which the GFP transgene remained silenced (Fig. 3.3A). Interestingly, the PVX vector alone reproducibly caused silencing reversal specific to stomatal guard cells in both immature and mature plants, suggesting that PVX affects silencing maintenance in this but no other cell type examined.

A total of three experiments were carried out with immature and mature plants, and one additional experiment was performed only with mature plants. Each experiment included four plants per treatment. Leaves showing obvious PVX symptoms and 3-4 leaves below the crown were harvested and one or two tissue sections were taken from each. A tissue section was considered positive if a field of view from the section showed GFP expression in cells other than guard cells, either in a widespread (>50%) pattern, or in large patches (as seen in PVX::HC-Pro infected plants, for example).

In immature plants, PVX::HC-Pro, PVX::AL2, PVX::AL2_{1-114}, and PVX::L2 all showed clear evidence of reversing PTGS directed against GFP (Fig. 3.3A). When examined under UV light, PVX::HC-Pro caused yellow-green GFP fluorescence to appear in isolated patches of cells, whereas PVX expressing the geminivirus proteins elicited GFP expression in a more evenly dispersed pattern. Similar patterns were apparent in >75% to 100% of the tissue sections examined in all experiments with all recombinant viruses, with the exception of one experiment where PVX::L2 was positive in ~50% of the sections. Northern blot analysis of GFP expression confirmed these observations (Fig. 3.3B).
In mature plants, however, only PVX::HC-Pro and PVX::AL2 were found to elicit significant expression outside of guard cells (Fig. 3.3A). As in immature plants, GFP expression was observed in >75% to 100% of the sections examined in each of four experiments. By contrast, GFP expression outside of guard cells was never observed with PVX::AL2_{1-114} and in only one of the four experiments with PVX::L2-inoculated plants, and in this case less than 50% of sections were positive. We concluded that AL2 can reverse GFP-directed silencing in both immature and mature leaves, whereas L2 and transcriptionally inactive AL2 can reverse silencing only in immature leaves. Given that a primary difference between immature and mature plants in these experiments was developmental age, this suggests that different mechanisms for PTGS maintenance may be active during different stages of development.

We next asked whether reducing methyl cycle activity by targeting the expression of ADK and SAHH could reverse silencing in immature plants. This study took advantage of Tobacco rattle virus (TRV) as a VIGS (virus induced gene silencing) vector (Ratcliff et al., 2001). The construction of TRV::ADK and TRV::SAHH has been described. These recombinant viruses are functional because they elicit severe symptoms in N. benthamiana (TRV alone is asymptomatic) and cause the reversal of TGS (Buchmann et al., 2008). Surprisingly however, they were unable to reverse PTGS in immature plants (not shown). Thus L2 (and AL2_{1-114}) activity was not phenocopied by treatments that reduce methyl cycle activity in this PTGS reversal assay. These results open the possibility that a second transcription-independent mechanism might exist, about which we have no further knowledge at present.
3.2.4 Summary and Conclusions

In summary, the studies reported here further extend our knowledge of AL2 and L2-silencing suppression and shed new light on the mechanisms themselves. First, we demonstrated that SAHH activity is needed for local silencing, providing further support for the idea that AL2 and L2 can interfere with the onset of local silencing by a transcription-independent mechanism that involves ADK and methyl cycle inhibition. In contrast, we found that inhibiting the methyl cycle had no impact on systemic spread of silencing and that AL2, but not L2, can prevent spread by a mechanism that likely involves transcriptional activation of silencing regulatory proteins. We also demonstrated that while AL2 and L2 can reverse established silencing in immature plants, only AL2 is able to do this in mature plants after flowering. This suggests that alternative silencing maintenance pathways are used at different stages of development, and that transcription-independent mechanisms are insufficient to inhibit the pathway that is active in mature plants.

To conclude, studies of AL2 reveal it to be a remarkably versatile PTGS suppressor that uses both transcription-dependent and -independent mechanisms to inhibit local silencing and subsequent systemic spread, as well as reversing established silencing in immature and mature plants (Trinks et al., 2005; van Wezel et al., 2002; Vanitharani et al., 2004; Voinnet et al., 1999; Wang et al., 2005; Yang et al., 2007). L2 is limited to transcription-independent mechanisms and shares many of these activities. In addition, both AL2 and L2 have recently been shown to reverse TGS (Buchmann et al.,
2008). The multiple aspects of RNA silencing suppressed by AL2 and L2, and the
discovery of additional silencing suppressors encoded by geminiviruses and their
satellites, emphasizes the critical role that silencing pathways play in modulating
geminivirus pathogenicity (Bisaro, 2006; Vanitharani et al., 2005). Much will be learned
about these pathways as we further unravel the mechanisms by which they are suppressed
by geminivirus proteins.
Figure 3.1 RNAi of SAHH suppresses local PTGS.

Transgenic (16c) *N. benthamiana* leaf tissues were coinfiltrated with *Agrobacterium* cultures delivering binary plasmids expressing GFP and one of the following: GUS, p19, TGMV AL2, or an RNAi construct against the methyl cycle enzyme SAHH (dsSAHH).

(A) Representative leaves were photographed under UV light 7-10 days postinfiltration. Under these conditions, GFP appears green, and the uniform dark red background is caused by chlorophyll autofluorescence. The negative control was infiltrated with GFP and GUS, and the positive control GFP and the silencing suppressor p19.

(B) Analysis of GFP mRNA by Northern Blot. Total RNA was isolated from infiltration zones 7 or 10 days postinfiltration (d.p.i.) and subjected to RNA gel blot hybridization. 5 µg of total RNA is gel-fractionated on an agarose-formaldehyde gel, and a photograph taken under UV light, with the 18S rRNA band used as a loading control. The gel was then blotted to positively charged nylon membrane, and probed with $^{32}$P-labeled GFP antisense riboprobe.

(C) SAHH mRNA levels were analyzed by RT-PCR. 500 pg of diluted total RNA from either GFP+GUS or GFP+SAHH infiltration patches was tested with an Invitrogen one-step RT-PCR kit, using primers derived from *N. tabacum* SAHH. 18S rDNA primers were added to the same reactions as a loading control.
Figure 3.1 RNAi of SAHH suppresses local PTGS
**Figure 3.2** AL2, but not L2, blocks the systemic silencing signal. Transgenic 16c *N. benthamiana* plants were agroinfiltrated with a mix of cultures containing binary plasmids for a GFP RNAi construct (dsGFP) and a putative systemic silencing blocker, either a protein or another dsRNA construct. GUS was used as a negative control and p19, shown previously to block systemic silencing, as a positive control. Top: After 4-6 weeks, evidence of systemic silencing was observed at the crown of the plant, and plants not showing evidence of systemic silencing were counted. Over two or more experiments, total “silencing escape” plants were tallied and the percentage graphed. Error bars represent the range between individual experiments. Bottom: An illustration of systemic silencing progression, from pre-infiltration to a fully silenced leaf.
Figure 3.2 AL2, but not L2, blocks the systemic silencing signal.
Figure 3.3 AL2 and L2 reverse established PTGS in a developmentally-dependent manner. 16c N. benthamiana plants were grown for 2 weeks then agroinfiltrated with dsGFP to trigger systemic silencing. 4-5 weeks after planting, they either were transferred to larger pots to keep them at vegetative stage of growth or allowed to bolt, and checked from 6-8 weeks for complete silencing (indicated by the entire plant showing red under UV light). The plants were then infiltrated with agrobacterium containing a recombinant PVX cDNA vector. The PVX either was an empty vector, contained HC-Pro (a protein known to reverse silencing), AL2, the mutant AL2\textsubscript{1-114}, or L2.

(A) 2-4 weeks after infiltration, tissue sections from older, symptomatic leaves were observed under a confocal microscope at 20x magnification. Deep red indicates chlorophyll autofluorescence and green indicates GFP expression.

(B) Analysis of GFP mRNA from systemic tissue. As before, total RNA was extracted from symptomatic tissue and probed with \textsuperscript{32}P-GFP antisense probe in a Northern Blot. Loading controls were EtBR stained 18S rRNA bands, with a picture taken prior to transfer to the membrane.
**Figure 3.3** AL2 and L2 reverse established PTGS in a developmentally-dependent manner.
CHAPTER 4

DISCUSSION

4.1 AL2 and L2 act as anti-DNA methylation proteins

4.1.1 AL2 and L2 can suppress TGS of both transgenes and endogenous genes

In this thesis, it was shown that both AL2 and L2 could reverse the transcriptional gene silencing of a GFP transgene in *N. benthamiana*, and of endogenous genes and repeats in *Arabidopsis*. This is to our knowledge the first example ever found of a viral protein capable of reversing TGS and genomic methylation. It was previously shown that a key feature of both AL2 and L2 was the inhibition of adenosine kinase, an enzyme associated with the methyl cycle pathway (Figure 1.5) (Wang et al., 2005; Wang et al., 2003). This led to the hypothesis that since both AL2 and L2 inactivates ADK they would also interfere with global methylation and transcriptional gene silencing. This was also bolstered by recent findings showing that methylation of both DNA and histones played an important part in defense against geminiviruses (Raja et al., 2008). We used a
PVX recombinant virus system and transgenically silenced 16c *benthamiana* to look at the effects of AL2, the mutants AL2<sub>1-114</sub> and AL2-C33A, and L2. All showed strong reversal of TGS, showing that TGS suppression does not require transcriptional activation. To further link the idea that ADK is required for TGS maintenance, we also used a Tobacco Rattle Virus (TRV) VIGS vector against the maintenance enzyme MET1 and the two methyl cycle enzymes SAHH and ADK. As predicted, silencing of all three enzymes caused the reversal of TGS, though ADK produced less robust GFP expression than the other two, owing probably to its roll as an ancillary rather then a primary enzyme. We then looked at endogenous gene silencing using dex-inducible transgenic *Arabidopsis* expressing AL2, AL2<sub>1-114</sub>, and L2. RT-PCR of several endogenous genes and pseudogenes (F-box protein, the SINE element AtSN1, the retrotransposon Athila and a CACTA-like DNA transposon) revealed that all three proteins also suppressed TGS of endogenous genes. The transcription of three of the four was enhanced in an activation-independent manner, though intriguingly the CACTA-like transposon’s transcription was only enhanced with the transcriptionally active AL2, indicating both an activation-dependent and activation-independent mechanism. When RT-PCR was performed on the same four loci in a transgenic dsADK knockdown plant, the results phenocopied the AL2<sub>1-114</sub> and L2 results. This showed that ADK inactivation was necessary and sufficient to reactivate transcription of some endogenous genes, but not others. Why did CACTA require a transcription-dependent mechanism to enhance transcription? CACTA, unlike the other three loci tested, is active in a *met1* mutant background, whereas the others become active in mutants that target non-CG methylation
(Tran et al., 2005; Zhang et al., 2006). So it is possible that CG methylation is more robustly maintained than non-CG methylation, and less vulnerable to a methyl cycle knockdown phenotype. This suggests in turn that wild type AL2 may enhance the transcription of an endogenous demethylase, such as the DEMETER (DME) family, REPRESSOR OF SILENCING 1, or an unknown antagonist to MET1 (Figure 4.1) (Penterman et al., 2007), though much work remains to be done.

4.1.2 AL2 and L2 reduce genomic methylation

To investigate whether or not AL2 and L2 also affect global methylation we looked at individual methyl-sensitive restriction sites within the studied loci, did bisulfite sequencing of part of the AtSN1 sequence, and used a methylation-sensitive cytosine extension assay to look at global CNG methylation in the genome. We found evidence of reduction of methylation at individual methyl groups, significant reductions of CNG and CHH methylation within AtSN1, and large reductions of global methylation (1.5-2.6 increase in counts) in plants expressing both AL2 and L2, showing an activation-independent mechanism for demethylation. This compliments the findings by Raja et al (Raja et al., 2008). That study showed hypermethylation in BCTV genomes in L2− mutants taken from secondary tissue, whereas wild type BCTV had much lower levels of methylation. That study also showed clearly that Arabidopsis defective in either DNA or histone methylation had much more severe pathogenesis when infected by geminiviruses than wild type plants, clearly showing methylation as a host defense against viruses, and giving a selective advantage for geminiviruses to suppress methylation and TGS as they
suppress PTGS. In addition, however, the question whether histone methylation is also affected by AL2 and L2 also should be looked at. The results from Raja et al strongly suggest that repressive marks on histones also plays a part in epigenetic defense, so it would be logical to assume that AL2, L2, or both might interfere with the maintenance of said marks.

4.2 Could animal viruses encode TGS suppressors?

The question of whether or not animal viruses might also encode TGS suppressors is more complex then plant viruses. In animals, the presence of unmethylated DNA is a strong trigger of innate immunity through detectors such as Toll-Like Receptor 9 (Hoelzer et al., 2008). However, mammalian cells, like plant cells, also utilize CG methylation as a way of silencing foreign DNA such as transposons and proviruses (Singal and Ginder, 1999), which presents a dilemma to incoming animal viruses. Some viruses with a latent stage in actively dividing cells, such as papillomaviruses and gamma herpesviruses, appear to take advantage of CG-methylation by becoming hypermethylated during the latent stage. When they enter their lytic cycle, they then become hypomethylated or even unmethylated (Tao and Robertson, 2003; Winocour et al., 1965). The Iridovirus family is even capable of functional levels of gene expression despite hypermethylation of its genome (Hoelzer et al., 2008). This would suggest that in many cases, DNA viruses in mammals may rely on suppression of innate immunity rather then suppression of DNA methylation, but there is some indirect evidence that viral genes devoted to demethylation may exist. For one, demethylation appears to be associated
with some viruses, such as herpesviruses, changing over from a latent to a lytic replication cycle. Another is that some oncogenic viruses, such as EBV, can cause changes (mostly hypermethylation) within the host’s epigenome, leading in some cases to transformation and cancer (Flanagan, 2007; Hoelzer et al., 2008). While it certainly is possible for animal viruses to encode proteins that perturb DNA methylation, so far the question of whether or not they produce proteins directly capable of suppressing TGS remains an open one.

4.3 AL2 and L2 have differing effects on initiation and reversal of systemic PTGS

4.3.1 AL2, but not L2, can suppress systemic PTGS

In this thesis we extended the studies that showed both AL2 and L2 could suppress local PTGS to systemic studies. By using 16c plants and dsGFP + test construct two-component infiltrations, we could observe the effects of different proteins on whether or not systemic silencing was initiated. Unlike the suppression of local PTGS, systemic PTGS suppression appeared to be transcriptional-activation dependent. AL2 protein provided nearly complete suppression of silencing, whereas AL2_{1\text{-}114} and L2 did not suppress systemic silencing above background. AL2-C33A, a partially activation-competent mutant, also gave partial suppression of systemic silencing. RNAi constructs for the methyl-cycle enzymes SAHH and ADK also failed to suppress systemic silencing, which is in line with the AL2 mutant results showing that inactivation of ADK is not
sufficient to suppress systemic silencing. These results are consistent with previous results with MYMV AC2, which also could prevent the spread of the systemic signal in an activation-dependent manner (Trinks et al., 2005). We concluded that AL2, like the old world AC2, was capable of blocking the spread of the systemic PTGS signal.

4.3.2 AL2 and L2 reverse established PTGS in a manner dependent on the developmental stage of the plant

In addition to blocking the systemic silencing signal, both AL2 and L2 were shown to be capable of reversing established PTGS as well. We first silenced 16c N. benthamiana plants with a dsRNA construct against the GFP transgene and then infected them with recombinant PVX viruses expressing AL2, AL2\textsubscript{1-114} or L2, while they were still in the vegetative (non-flowering) stage. When examined under a confocal microscope, plants infected with all three viruses showed evidence of GFP expression, which was confirmed by mRNA via Northern Blot, showing that both AL2 and L2 were capable of reversing silencing at this stage of a plant’s development. However, when bolting and flowering (mature) plants were infected with the same viruses, only AL2 showed frequent expression of GFP under the microscope; L2 reversed silencing on a much less frequent basis then in immature plants, confirmed by mRNA analysis. This intriguing finding raises the possibility that two separate maintenance mechanisms exist for PTGS, depending on the developmental stage of the plant.
4.3.3 The role of DNA methylation in PTGS

Very little is known to date about the mechanisms to maintain PTGS silencing once it has established itself. It has long been known that DNA methylation was associated with PTGS (Jones et al., 1999; Llave et al., 2000). The RNAi knockdown of methyl-cycle enzymes also suppresses local PTGS silencing, providing further evidence of the importance of DNA methylation for at least one stage of PTGS. However, when silenced 16c plants were infected with TRV VIGS constructs against either a maintenance enzyme (MET1) or methyl-cycle enzymes SAHH or ADK, no evidence of PTGS reversal is present. Further, the knocking down either SAHH or ADK does not suppress the initiation of systemic silencing. This would seem to imply that though methylation may be important for cell-dependent or perhaps short-distance silencing, it is not involved in either initiation or maintenance of systemic PTGS. How might it be involved in local PTGS? That is unclear as of yet, though one possibility is that the methylation of the coding region itself produces siRNA, similar to what has been shown for regions of heterochromatin, giving a further boost to the siRNA signal (Lister et al., 2008; Zhang et al., 2006) (Figure 4.1). Though methylation does not appear to be directly important in the establishment or maintenance of systemic PTGS, more information on the nature of the silencing signal may help further shed light on this pathway.
4.3.4 AL2 mechanism of PTGS reversal from vegetative to flowering plants

The result of the data is a significantly more complicated picture of the mechanism for AL2 action, since AL2 suppresses PTGS in an activation-independent manner at the local level and blocks the spread of the systemic signal in an activation-dependent manner. After systemic silencing is established in an immature plant it once more can reverse it in an activation-independent manner, but requires transcriptional activation to reverse it in a flowering plant. One possibility of how AL2’s mechanism of action changes over a plant’s development concerns recent findings showing that the ACMV protein AC2 causes an increase in transcription of a putative endogenous silencing suppressor, WEL1. In addition to WEL1, there are other proteins that have been identified as endogenous silencing suppressors, such as FIREY1, XRM2 and XRM3, which all work by distinct mechanisms (Gazzani et al., 2004; Gy et al., 2007). One possibility is an endogenous protein – WEL1 or another endogenous suppressor – has its transcription enhanced, which in turn interferes with a maintenance step and leads to the breakdown of systemic silencing. While this seems both necessary and sufficient in flowering plants, it appears not necessary in immature plants (though it may still be sufficient, as AL2 still produced a stronger overall silencing reversal then either the transcriptionally active mutant AL2_{1,114} or L2). Other possibilities exist as well. A change in the stability of either endogenous proteins or the viral proteins as the plant begins to flower, for example, may also account for the altered silencing suppression properties. Much work remains to be done to being to elucidate these differing
mechanisms of maintenance. Overall, what we know about AL2 mechanisms of action and systemic silencing maintenance is shown in Figure 4.1.
Figure 4.1 A new model for AL2 silencing suppression activities.

Geminivirus AL2 and L2 proteins, as previously shown, both interact with and inactivate ADK. This in turn suppresses the methyl cycle due to the buildup of waste adenosine and in turn causes the maintenance of methylation to partially break down, resulting in the reversal of endogenous TGS and a reduction of methylation within the genome. AL2 also enhances the transcription of a number of host proteins, including WEX1 and WEL1, which may act as endogenous suppressors of silencing. AL2, but not L2, can block the spread of the systemic silencing signal, related to its ability to activate transcription, though by what mechanism is not yet clear. Both AL2 and L2 can reverse established systemic silencing in immature (vegetatively growing) N. benthamiana, but only transcriptionally-active AL2 can reverse systemic silencing in mature (flowering) plants. This suggests two different maintenance mechanisms for systemic PTGS, the specifics of which are not yet known.
Figure 4.1 A new model for AL2 silencing suppression activities.


