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Adenosine Kinase Inhibition and Suppression of RNA Silencing by Geminivirus AL2 and L2 Proteins

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Most plant viruses are initiators and targets of RNA silencing and encode proteins that suppress this adaptive host defense. The DNA-containing geminiviruses are no exception, and the AL2 protein (also known as AC2, C2, and transcriptional activator protein) encoded by members of the genus *Begomovirus* has been shown to act as a silencing suppressor. Here, a three-component, *Agrobacterium*-mediated transient assay is used to further examine the silencing suppression activity of AL2 from *Tomato golden mosaic virus* (TGMV, a begomovirus) and to determine if the related L2 protein of *Beet curly top virus* (BCTV, genus *Curtovirus*) also has suppression activity. We show that TGMV AL2, $AL2_{1\text{-}100}$ (lacking the transcriptional activation domain), and BCTV L2 can all suppress RNA silencing directed against a green fluorescent protein (GFP) reporter gene when silencing is induced by a construct expressing an inverted repeat GFP RNA (dsGFP). We previously found that these viral proteins interact with and inactivate adenosine kinase (ADK), a cellular enzyme important for adenosine salvage and methyl cycle maintenance. Using the GFP-dsGFP system, we demonstrate here that codelivery of a construct expressing an inverted repeat ADK RNA (dsADK), or addition of an ADK inhibitor (the adenosine analogue A-134974), suppresses GFP-directed silencing in a manner similar to the geminivirus proteins. In addition, AL2/L2 suppression phenotypes and nucleic acid binding properties are shown to be different from those of the RNA virus suppressors HC-Pro and p19. These findings provide strong evidence that ADK activity is required to support RNA silencing, and indicate that the geminivirus proteins suppress silencing by a novel mechanism that involves ADK inhibition. Further, since $AL2_{1\text{-}100}$ is as effective a suppressor as the full-length AL2 protein, activation and silencing suppression appear to be independent activities.

In eukaryotic cells, homology-dependent silencing that operates at the RNA level is involved in a number of fundamental processes, including cellular defense against viruses, control of transposon mobility, developmental gene regulation via microRNAs (miRNAs), de novo histone and DNA methylation, and the establishment of heterochromatin (8, 9, 60, 63). RNA silencing is triggered by double-stranded RNA (dsRNA), and a defining feature is the appearance of short interfering RNA (siRNA), 21- to 26-nucleotide (nt) dsRNA species homologous to the silenced gene (17, 68). These siRNAs are produced from inducing dsRNA by the action of RNase III-like enzymes called Dicer or Dicer-Like (12). In turn, the antisense strands of unwound siRNAs guide another RNase-containing complex, the RNA-induced silencing complex (RISC), to homologous single-stranded RNA (ssRNA) targets (usually mRNA) for degradation (19). Methylation of homologous nuclear DNA corresponding to transcribed regions also occurs, although the role of methylation in RNA silencing is unclear (3, 4, 26).

RNA silencing acts as an antiviral defense in both plant and animal (insect) cells, and viruses are both inducers and targets of the system and thus determine its specificity (31, 32, 55, 59, 65). To counter this adaptive defense, viruses from different families have elaborated a variety of apparently unrelated suppressor proteins that affect different, and possibly multiple, steps in the silencing pathway (33, 61). For example, P1/HC-Pro (HC-Pro) of *Tobacco etch virus* (TEV) and related potyviruses can reverse established silencing in plants and suppress local silencing of reporter genes in transient assays (2, 6, 27, 35). HC-Pro interacts with a cellular protein (rgs-CaM) that is itself a silencing suppressor, suggesting that the viral protein stimulates an endogenous regulatory pathway (1). It also at least partially inhibits dsRNA processing by Dicer (14, 37). In addition, HC-Pro appears to block unwinding of siRNA/siRNA* duplexes, thereby preventing the incorporation of targeting information into RISC (10). In contrast, the p19 protein of *Cymbidium ringspot virus* and related tombusviruses cannot reverse established silencing, although it blocks the production of a systemic silencing signal in plants and can suppress local silencing in transient assays. The activity of p19 is due to its ability to bind and sequester siRNAs, which could also prevent incorporation of siRNA into RISC (29, 46, 58, 67). Interestingly, HC-Pro and p19 impact both siRNA and miRNA metabolism, underscoring the similar and overlapping natures of these pathways (10, 14, 28). Further study of HC-Pro, p19, and other viral suppressor proteins will no doubt provide additional insight into the molecular mechanisms of RNA silencing and related processes.

The geminiviruses package ssDNA that replicates in the host cell nucleus through dsDNA intermediates that assemble into minichromosomes (15, 20, 41). Geminiviruses do not encode

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polymerases but specify multifunctional proteins that provide a cellular environment favorable to replication, initiate specific steps in replication and/or transcription, potentiate virus spread within and between hosts, and suppress host defenses. For example, the AL2 protein of *Tomato golden mosaic virus* (TGMV; genus *Begomovirus*) is a transcription factor required for the expression of late viral genes (49–51). The 15-kDa AL2 protein (also known as AC2, C2, or transcriptional activator protein) has a C-terminal activation domain that is functional in plant, yeast, and mammalian cells (22). AL2 is also a pathogenicity factor, and homologues from several begomoviruses have been shown to reverse RNA silencing in plants and to suppress local silencing in transient assays (56, 57, 61). In addition, TGMV AL2 and the related L2 protein of *Beet curly top virus* (BCTV; genus *Curtovirus*) condition a virus-nonspecific enhanced-susceptibility phenotype in transgenic plants which is attributable to their ability to inactivate SNF1-related kinase (21, 52). AL2 and L2 also interact with and inactivate adenosine kinase (ADK), which phosphorylates adenosine to produce 5′-AMP (64). Because AMP can stimulate SNF1 activity, the inactivation of SNF1 and ADK by AL2/L2 may represent a dual mechanism to counter SNF1-mediated antiviral responses.

ADK is generally considered to be a housekeeping enzyme involved in adenosine salvage. More recently, it has also been shown to play a key role in sustaining the methyl cycle and *S*-adenosylmethionine-dependent methyltransferase activity (30, 39, 45, 66). In yeast, methylation deficiency is the primary defect of ADK-null mutants. ADK deficiency also reduces methyltransferase activity in plants, and observational evidence suggests that this can compromise the maintenance of RNA silencing (39, 64).

In this report, a transient system is used to demonstrate that TGMV AL2 protein and the related L2 protein from the curtovirus BCTV can suppress RNA silencing directed against a green fluorescent protein (GFP) reporter gene. We also demonstrate that inhibiting cellular ADK activity causes similar silencing suppression, providing evidence that the TGMV AL2 and BCTV L2 proteins counter RNA silencing by reducing ADK activity. We further show that the AL2 and L2 proteins operate by mechanisms which differ from those of HC-Pro and p19.

MATERIALS AND METHODS

Delivery of RNA silencing targets, inducers, and suppressors. *Agrobacterium tumefaciens*-mediated transient expression in *Nicotiana benthamiana* plants or *N. benthamiana* line 16c containing a GFP transgene (44) (provided by David Baulcombe, Sainsbury Laboratory) was based on the three-component system described by Johansen and Carrington (25). Equal volumes of *A. tumefaciens* cultures (optical density at 600 nm = 1) harboring plasmids designed to express the silencing target (GFP), the silencing inducer (an inverted repeat GFP RNA [dsGFP]), or a silencing suppressor or control were mixed and coinfiltrated to the underside of leaves using a 1-ml syringe. In some experiments, the ADK inhibitor A-134974 (38) (Sigma) was mixed with bacterial cultures to final concentrations of 0.01 to 100 μM just prior to infiltration. GFP fluorescence was observed using a 100-W, longwave UV lamp (Blak-Ray Model B 100YP; UV Products). Photographs were taken with UV and yellow filters and uniformly processed using Adobe Photoshop. Tissue was harvested from infiltration zones and used for RNA and protein isolation. RNA analysis is described below. ADK activity levels in protein extracts were measured as described previously (64).

Plasmids. In most cases, expression was driven from a pRTL2-derived cassette consisting of the enhanced *Cauliflower mosaic virus* 35S promoter, the TEV 5′ nontranslated leader, and the 35S terminator (43). Constructs containing β-glucuronidase (GUS), GFP, dsGFP, and HC-Pro were provided by James Carrington (Oregon State University) (25). Plasmids containing *African cassava mosaic virus* (ACMV) AC2, TGMV AL2 and $AL2_{1-100}$, and BCTV L2 were generated from earlier constructs (52) by inserting the genes into pRTL2 as NcoI-BglII fragments. Ti plasmid constructs were prepared by inserting the expression cassettes into HindIII-BamHI-digested pKJB5033 (a derivative of pBI121; Clontech; K. J. Buckley, unpublished). pBIN61-p19 was obtained from David Baulcombe (62).

The dsADK plasmid contained ~500 bp of *Arabidopsis* ADK2 (nt 306 to 814) (64) in the sense and antisense orientations separated by an intron containing GUS sequence and was expressed from the 35S promoter in pFGC1008 (47) (www.chromdb.org/plasmids/vectors2.html). ADK sequence was amplified by PCR from a cDNA library obtained from the Arabidopsis Biological Resource Center (Ohio State University). The 5′ primer contained SpeI (italics) and AscI (underlined) restriction sites (5′-GAG*ACTAGT*GGCGCGCCGGATGCTACA GCAGCTGG). The 3′ primer contained BamHI (italics) and SwaI (underlined) restriction sites (5′-GAG*GGATCC*ATTTAAATCCACTGGATCAGCGCCC TG). DNA fragments were digested with SpeI and BamHI or AscI and SwaI and sequentially inserted into pFGC1008. The dsSNF1 construct contained an SmaI-XbaI fragment from *Arabidopsis* SNF1 (AKIN 11; nt 1 to 1,028) (21) in the sense and antisense orientations separated by the GUS intron from pFGC1008 and was expressed from the 35S promoter in pBI121.

RNA analysis. RNA was obtained using TRIzol reagent (Invitrogen). For mRNA detection, 15 μg was fractionated on formaldehyde-agarose gels and transferred to nitrocellulose. For siRNA detection, 45 μg was separated by electrophoresis in 15% polyacrylamide gels containing 7 M urea in Tris-borate-EDTA buffer (pH 8.0), transferred to Hybond-NX membrane (Amersham), and UV cross-linked (1,200 μJ; Stratalinker; Stratagene). Probes were prepared by in vitro transcription using the Strip-EZ RNA kit (Ambion) in the presence of [α-^{32}P]UTP. Antisense GFP probes were used to detect GFP mRNA, and probes of sense and antisense polarities were used separately or together for analysis of GFP-derived siRNA.

The Superscript One-Step RT-PCR kit (Invitrogen) was used both to clone and to detect SNF1 and ADK mRNA sequences from total cellular RNA obtained from *N. benthamiana* tissue. First, *N. benthamiana* ADK and SNF1 cDNA fragments were generated using a primer set designed from *Arabidopsis* ADK2 sequence (nt 223 to 860; 5′ primer, 5′-ATGGGATCCATTGGAAAGGAC; 3′ primer, 5′-TCACCTGCACCGTTGGTG) or from *Arabidopsis* SNF1 sequence (AKIN11, nt 598 to 1299; 5′ primer, 5′-GATGTATGGAGTTGCGG; 3′ primer, 5′-CCATCGACATTTCATGTT). The *N. benthamiana* ADK cDNA (GenBank accession number AY741533) and SNF1 cDNA (GenBank accession number AY919676) fragments were cloned into a T-vector (pKJB5048; Buckley, unpublished) and the sequences used to design primers for reverse transcription (RT)-PCR detection of ADK or SNF1 mRNA in RNA preparations from infiltration zones. Primers for either ADK (5′ primer, 5′-GGGAGAAAATGAAGAACAA TGC; 3′-GGCAACGGTATTACAGGGAAC) or SNF1 (5′ primer, 5′-GCTCT TCTCTGTGGCACCCTTC; 3′-CACACATTTAGTTCTTGCAGAGC) and 18S rRNA (internal control) were used in each reaction. The rRNA primer set was derived from *Arabidopsis* 18S rRNA sequence (nt 3 to 1293; 5′ primer, 5′-CCTGGTTGATCCTGCCAGTAG; 3′ primer, 5′-ACCAACTAAGAACGG CCATGC). Equivalent samples, normalized by the amount of 18S rRNA product, were loaded on agarose gels and subjected to DNA gel blot hybridization analysis using *N. benthamiana* ADK or SNF1 sequences as probes. Probes were prepared using the Rediprime II random primer labeling system (Amersham) with [α-^{32}P]dCTP.

Mobility shift analysis. Expression and purification of a glutathione *S*-transferase-AL2 fusion protein (GST-AL2) from *Escherichia coli* has been described previously (22). A GST-p19 expression construct was provided by Herman Scholthof (Texas A&M University), and GST-p19 protein and GFP siRNA were gifts from Yijun Qi, Xuehua Zhong, and Biao Ding (Ohio State University). The dsRNA starting material used to prepare the siRNA was generated by annealing in vitro transcription products synthesized from sense and antisense GFP strands in the presence of [α-^{32}P]UTP. The internally labeled dsRNA was incubated in wheat germ extract essentially as previously described (53). Small RNA products were analyzed by electrophoresis in 15% polyacrylamide gels containing 8 M urea, and siRNA bands were eluted. The resulting siRNA probe was incubated with 1 μg GST, GST-p19, or GST-AL2 in 10 mM Tris-HCl (pH 7.5)–5 mM $MgCl_2$–50 mM NaCl–66 mM KCl–5 mM dithiothreitol for 20 min at room temperature.

A DNA fragment (~500 bp) from pUC18 was end labeled using [γ-^{32}P]ATP and T4 DNA kinase. Labeled DNA was separated from unincorporated nucleotides by Sephadex G-50 chromatography, boiled for 5 min, and quick cooled on ice. The resulting ssDNA probe was incubated with 1 μg GST or GST-AL2 in 10

mM Tris-HCl (pH 7.5)–1 mM $MgCl_2$–50 mM NaCl–0.5 mM EDTA–0.5 mM dithiothreitol–40 μg/ml poly(dI-dC) for 20 min at room temperature. Protein-nucleic acid complexes were resolved by electrophoresis in nondenaturing 5% polyacrylamide gels in Tris-borate-EDTA buffer at 4°C and visualized using a phosphorimager (Bio-Rad Molecular Imager FX).

RESULTS

RNA silencing is suppressed by TGMV AL2 and BCTV L2 proteins. We set out to confirm that TGMV AL2, like its counterparts from begomoviruses such as ACMV, can suppress RNA silencing and to determine whether the AL2 transcriptional activation domain is necessary for this activity. We also asked whether the related BCTV (curtovirus) L2 protein, which unlike AL2 is not required for late viral gene expression, can also suppress RNA silencing (24, 48). This study employed a transient system that involves coinfiltration of *N. benthamiana* leaves with *A. tumefaciens* cultures harboring Ti plasmids that express GFP, an inverted repeat GFP RNA as a strong silencing inducer (dsGFP), and a test construct or control (25). Constructs expressing HC-Pro, p19, and ACMV AC2 (AL2) were used as positive controls, and a construct expressing GUS was a negative control. GFP expression in infiltration zones could be visualized under UV light as green or yellow fluorescence against a red background of chlorophyll fluorescence. The accumulation of GFP mRNA and silencing-derived, GFP-specific siRNAs in infiltrated tissues was assessed by RNA gel blot hybridization.

We found that AL2 and L2 can suppress GFP-directed silencing in the transient system, as judged by the appearance of green or yellow fluorescence (Fig. 1A) and by the increased accumulation of GFP mRNA in infiltration zones relative to the GUS negative control (Fig. 1B). However, GFP fluorescence was less intense with the geminivirus proteins than with HC-Pro and p19 (Fig. 1A). $AL2_{1\text{-}100}$, which lacks the transcriptional activation domain, suppressed silencing to about the same degree as full-length AL2. Consistent with visual inspection, the greatest GFP mRNA accumulation was observed with p19, while HC-Pro also supported robust but somewhat lower accumulation of this transcript. The geminivirus proteins behaved as weaker suppressors by comparison (Fig. 1B). However, they proved about as effective as p19 in reducing the accumulation of GFP-specific siRNAs over the 5-day course of the experiments (Fig. 1C). That these species are dsRNA was indicated by their ability to hybridize with probes of sense and antisense polarities (data not shown). In contrast, GFP-derived siRNA accumulated to relatively large amounts after 5 days in the HC-Pro and GUS treatments, although GUS did not support GFP mRNA accumulation. The same results were obtained in four independent experiments using RNA preparations from infiltrated wild-type *N. benthamiana* or *N. benthamiana* line 16-C plants, which express a GFP transgene (44). However, it should be noted that relatively weak activity coupled with inherent experimental variability precluded a precise ordering of the relative abilities of geminivirus proteins to suppress silencing. Our results with the positive control proteins HC-Pro, p19, and AC2 were similar to those obtained previously by others, although some differences in siRNA accumulation have been observed in different studies (see Discussion).

We concluded that TGMV AL2 and the related BCTV L2 protein similarly suppress silencing directed against a reporter gene, although their activity can be characterized as weak in comparison to p19 and HC-Pro. Further, since $AL2_{1\text{-}100}$ is as effective a suppressor as full-length AL2 protein, the activation and silencing suppression activities of AL2 appear to be independent.

RNA silencing is suppressed by an ADK inverted repeat construct. We next asked whether reducing expression of the two cellular kinases known to be inhibited by AL2 and L2 affected GFP silencing. We adopted an RNA interference approach to explore this question, based on the observation that components of the silencing machinery, and by analogy other proteins that might play a supporting role, can be at least partially silenced (5). Inverted repeat constructs designed to express dsRNA were prepared using *Arabidopsis* ADK2 (dsADK) and SNF1 kinase AKIN11 (dsSNF1) sequences. ADK and SNF1 genes are highly conserved among plants, mammals, and yeasts, and both of the *Arabidopsis* proteins can functionally complement corresponding yeast mutants (21, 64). Thus, it was considered likely that these sequences would trigger silencing directed against *N. benthamiana* ADK and SNF1. Subsequent measurements of ADK and SNF1 mRNA levels in infiltrated tissue showed that this occurred (see below).

As was apparent from GFP fluorescence and RNA gel blot assays, expression of dsADK as the third component of the transient system (GFP, dsGFP, and dsADK) suppressed GFP-directed silencing by supporting the accumulation of GFP mRNA and reducing the accumulation of GFP siRNA in a manner similar to the geminivirus proteins. In contrast, the dsSNF1 construct failed to suppress GFP silencing when used as the third component (Fig. 1).

We confirmed that the dsADK and dsSNF1 constructs reduced their target mRNA levels by using RT-PCR to examine RNA extracts from infiltrated tissue. PCRs contained ADK- or SNF1-specific primer pairs designed from cloned *N. benthamiana* cDNA fragments, as well as a primer set to amplify 18S rRNA as an internal control. Nucleotide sequence comparisons revealed extensive identity between the cloned ADK and SNF1 fragments from *N. benthamiana* and the corresponding *Arabidopsis* mRNAs (data not shown). As evident from the ethidium bromide-stained agarose gel shown in Fig. 2A, ADK mRNA levels were substantially reduced by the dsADK construct but remained unaffected by other treatments. DNA gel blot hybridization confirmed the identity of the PCR-generated ADK fragment (data not shown). A substantial reduction in SNF1 mRNA levels was likewise observed in tissue infiltrated with dsSNF1, but as noted previously, DNA gel blot hybridization was necessary to detect the PCR fragment corresponding to this low-abundance transcript, even in control tissue (21) (Fig. 2B).

Thus, reducing ADK mRNA levels, but not SNF1 mRNA levels, resulted in suppression of RNA silencing in the transient system. However, because SNF1-related kinases constitute a small gene family in most plant species (11, 16), we cannot rule out the possibility that other family members could functionally substitute for the one targeted by our dsSNF1 construct.

RNA silencing is suppressed by an ADK inhibitor. The results obtained with the dsADK construct prompted a search for another means to inhibit ADK. By employing an assay that

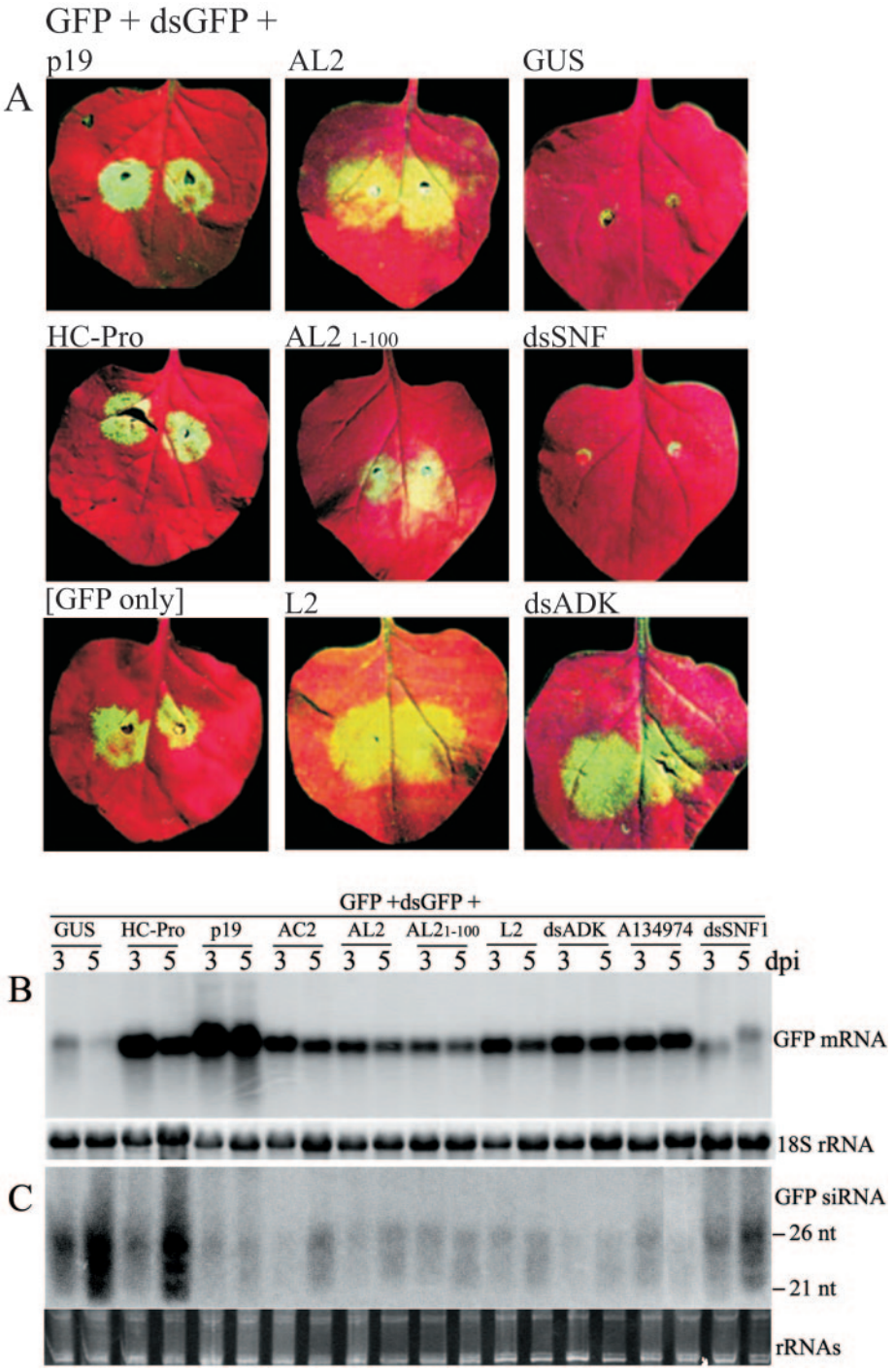

FIG. 1. Silencing suppression by AL2 and L2 proteins and treatments that reduce ADK activity. *N. benthamiana* leaf tissues were coinfiltrated with *Agrobacterium* cultures delivering Ti plasmids expressing GFP, dsGFP and the indicated proteins, or inverted repeat RNA constructs. One treatment included the adenosine inhibitor A-134974 as the third component. (A) Representative leaves were photographed under UV light 5 days postinfiltration. Each half-leaf was infiltrated, and under these conditions GFP fluorescence appears green or yellow. The GFP control (GFP only) was infiltrated with a mixture of *Agrobacterium* cultures containing the GFP-expressing construct and empty vector. (B and C) Analysis of GFP mRNA and GFP-derived siRNAs, respectively. Total RNA was isolated from infiltration zones 3 and 5 days postinfiltration (dpi) and subjected to RNA gel blot hybridization analysis. In panel B, agarose gel-fractionated RNA (15 μg) was hybridized with a ^{32}P-labeled antisense GFP riboprobe. In panel C, RNA (45 μg) was fractionated on a 15% polyacrylamide gel containing 7 M urea and hybridized with mixed-sense and antisense GFP riboprobes. The positions of 21- and 26-nt markers are indicated. rRNAs are shown as loading controls.

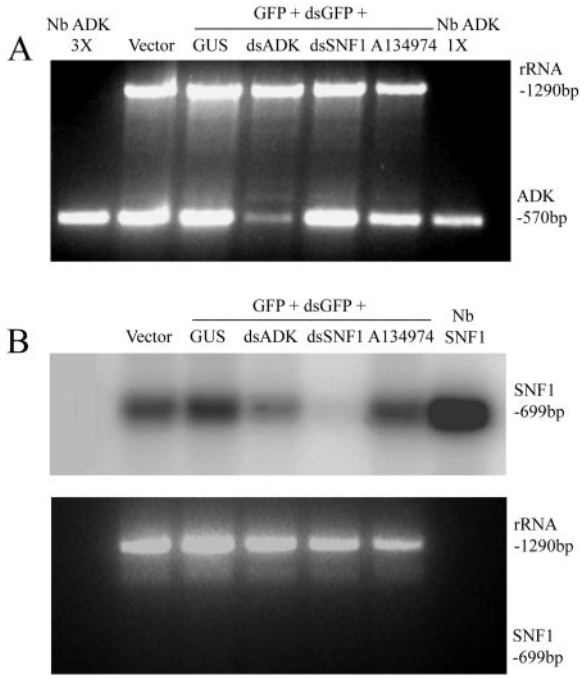

FIG. 2. Reduction of ADK and SNF1 mRNA levels by dsADK and dsSNF1 constructs. *N. benthamiana* leaf tissues were infiltrated with *Agrobacterium* cultures delivering Ti plasmids expressing GFP, dsGFP and the indicated proteins or vector control, inverted repeat RNA constructs, or the adenosine inhibitor A-134974 (100 μM). ADK mRNA or SNF1 mRNA in RNA extracts prepared from infiltration zones 5 days postinfiltration was detected by RT-PCR. (A) Reaction mixtures included ADK and 18S rRNA (internal control) primer sets, and products derived from ADK mRNA (570 bp) and rRNA (1,290 bp) were detected by ethidium bromide staining following agarose gel electrophoresis. The first and last lanes show an ADK marker fragment generated by PCR from a plasmid containing *N. benthamiana* ADK cDNA (Nb ADK). Three times as much product was loaded in the first lane compared to the last lane (3X and 1X). (B) Reaction mixtures contained SNF1 and 18S rRNA primer sets, and products derived from SNF1 mRNA (699 bp) were detected by DNA gel blot hybridization using a ^{32}P-labeled probe specific for SNF1 (top). The 18S rRNA products (1,290 bp), but not the SNF1 products, could be detected by ethidium bromide staining prior to gel blot analysis (bottom). The last lane shows an SNF1 marker fragment generated by PCR from a plasmid containing *N. benthamiana* SNF1 cDNA.

uses thin layer chromatography to measure adenosine phosphorylation in the presence of γ-^{32}P-ATP (64), it was determined that the adenosine analogue A-134974 (38), a selective inhibitor of rat ADK, also inhibits ADK activity in a dose-dependent manner when added to protein extracts from *N. benthamiana* plants (Fig. 3).

In subsequent experiments, coinfiltration of A-134974 with *Agrobacterium* cultures harboring GFP and dsGFP constructs (GFP, dsGFP, and A-134974) resulted in suppression of GFP silencing similar to the geminivirus proteins and dsADK. GFP mRNA was observed to accumulate in infiltrated tissue, whereas the accumulation of siRNAs derived from GFP sequences was reduced relative to the GUS negative control (Fig. 1B and C). Silencing suppression was observed over the range of A-134974 concentrations tested (0.01 to 100 μM), although some variability was observed at lower concentrations (data not shown).

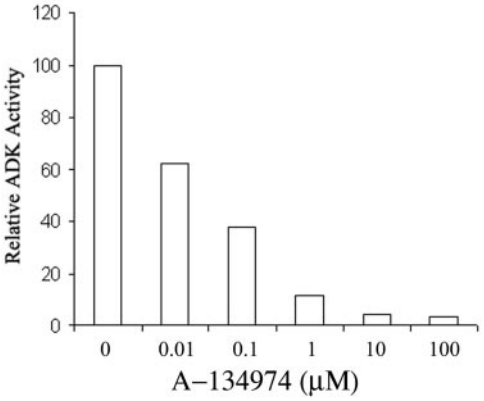

FIG. 3. The adenosine analogue A-134974 inhibits ADK activity in *N. benthamiana* extracts. Reaction mixtures contained the substrates adenosine (1 μM) and [γ-^{32}P]ATP, with 400 ng of total protein extract from stem tissue and the indicated concentrations of A-134974. It was previously determined that the level of ADK activity is proportional to the amount of added extract over a range of 100 to 500 ng (64). Labeled AMP and ATP were resolved by thin-layer chromatography, and ADK activity (AMP/AMP and ATP) in each reaction was calculated following phosphorimager quantitation of radioactivity in individual spots. The graph shows relative ADK activity plotted against increasing A-134974 concentrations.

Although A-134974 has been shown to be a selective inhibitor of rat ADK (38), its possible effects on plant enzymes other than ADK are not known. Nevertheless, it can be said that reducing ADK mRNA levels by introducing a dsADK construct, or reducing ADK activity by adding an enzyme inhibitor, leads to silencing suppression in this transient system (Fig. 1 and 2A). Together, these results indicate that ADK activity is needed either for the initiation of RNA silencing or for its maintenance over the 5-day course of the experiment.

ADK activity is reduced in infiltrated leaf tissues expressing AL2 or L2 protein and exhibiting silencing suppression. We previously showed that AL2 and L2 are effective inhibitors of ADK activity both in vitro and in vivo, including geminivirus-infected tissue (64). Thus, we asked whether suppression of RNA silencing by these proteins and other agents used in these studies could be correlated with reductions in ADK activity. ADK activity levels in protein extracts obtained from tissues infiltrated 5 days previously with various test constructs were determined as previously described (64). In tissues receiving GFP-dsGFP and AL2, L2, dsADK, or A-134974, we found that ADK activity was markedly reduced, by more than 50 to 90% (Fig. 4). Comparable tissues contained relatively high levels of GFP mRNA and low levels of GFP-specific siRNAs compared to the GUS negative control (Fig. 1B and C). In contrast, in tissues infiltrated with GFP-dsGFP and GUS, dsSNF1, or HC-Pro, ADK activity was not significantly reduced relative to control tissue infiltrated with vector DNA, regardless of whether the constructs did (HC-Pro) or did not (GUS, dsSNF1) suppress RNA silencing (Fig. 4 and 1B and C). Thus, silencing suppression by AL2, L2, dsADK, and A-134974 was strongly correlated with substantial reductions in ADK activity, and the inability of HC-Pro to significantly inhibit the activity of this enzyme indicates that this is not a general feature of tissues exhibiting silencing suppression.

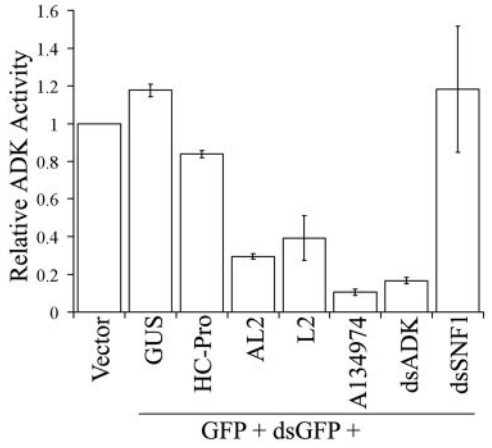

FIG. 4. ADK activity in infiltration zones. *N. benthamiana* leaves were agroinfiltrated with GFP, dsGFP and the indicated constructs, empty vector, or the adenosine analogue A-134974 (100 μM). Protein extracts were prepared from infiltrated tissue 5 days postinfiltration, and ADK assays were performed by incubating samples (250 ng) with adenosine and [γ-^{32}P]ATP. Products were fractionated by thin-layer chromatography, and labeled AMP was quantitated by phosphorimaging as described in the legend to Fig. 3. The graph shows ADK activity relative to the vector control. The data are averages of two experiments; the error bars indicate ranges.

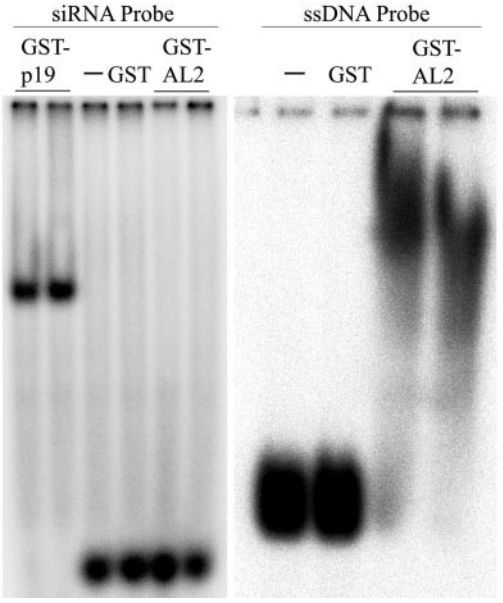

FIG. 5. AL2 does not bind siRNA. Autoradiographs of nondenaturing polyacrylamide gels illustrating electrophoretic mobility shift experiments with an siRNA probe (left panel) and an ssDNA probe (right panel) are presented. Labeled siRNA or ssDNA (~500 bp) was incubated without added protein (−) or with GST (negative control), GST-p19, or GST-AL2 in duplicate reactions as indicated. The inability of GST-AL2 to bind siRNA was confirmed twice with each of two different protein preparations.

AL2 does not bind siRNA. It has been demonstrated that some plant viral silencing suppressors, including p19 and *Beet yellows virus* p21, bind siRNA (and similar miRNA) duplexes (10, 46). The influenza virus NS1 protein also has siRNA binding activity (32). TGMV AL2 protein is known to bind ssDNA and dsDNA in a sequence-nonspecific manner, although the significance of this activity is unknown (22). The possibility that this orphan nucleic acid binding activity might extend to siRNA was directly tested.

Electrophoretic mobility shift experiments were carried out to assess the binding activity of recombinant GST-AL2 fusion protein expressed in *E. coli* and purified as previously described (22). Similarly prepared GST-p19 and GST served as positive and negative controls, respectively. The proteins were incubated with an siRNA population isolated from wheat germ extract primed with GFP-derived, ^{32}P-labeled dsRNA (53). In the absence of added protein or in the presence of GST or GST-AL2, the labeled siRNA migrated near the bottom of the gel, whereas in the presence of GST-p19 nearly all of it was found in slower-migrating complexes (Fig. 5). GST and GST-AL2 were additionally incubated with a labeled ~500-nt ssDNA fragment from pUC18 to verify biological activity of the viral fusion protein (22). This experiment demonstrated that the AL2 protein was active as GST-AL2, but not GST, and was able to form slower-migrating complexes with ssDNA (Fig. 5). Thus, AL2 does not have significant siRNA binding activity under conditions that support its efficient binding by the p19 protein.

DISCUSSION

We previously found that the AL2 and L2 proteins interact with and inactivate ADK in vitro and in vivo and that ADK activity is reduced in TGMV- and BCTV-infected tissue in an AL2/L2-dependent manner (64). In this report, we showed that the TGMV AL2 and BCTV L2 proteins similarly act as suppressors of RNA silencing directed against a GFP reporter gene in a transient three-component system and that silencing suppression does not require the AL2 transcriptional activation domain. Since the BCTV protein is not required for viral gene expression and does not activate transcription in yeast, it is likely that suppression by L2 likewise does not depend on transcriptional activation (21, 24, 48). We further demonstrated that reducing ADK mRNA levels by expression of an inverted repeat construct directed against ADK (dsADK), or reducing ADK activity by adding an inhibitor (A-134974), results in a similar suppression of RNA silencing. GFP mRNA accumulated to relatively high levels, and GFP-derived siRNAs were reduced in tissues infiltrated with GFP-dsGFP and AL2, $AL2_{1\text{-}100}$, L2, dsADK, and A-134974 compared with the GUS negative control. Direct measurement of ADK activity in comparable tissues following infiltration with these same constructs revealed that ADK activity was significantly reduced (>50% to 90%) relative to control tissue infiltrated with vector or GUS. Thus, ADK activity is required to initiate or maintain RNA silencing in the transient assay, and ADK inhibition by AL2 and L2 is at least part of the mechanism by which these related proteins suppress silencing.

It has been reported that mutational disruption of a likely nuclear localization signal of *Tomato yellow leaf curl virus-China* C2 (AL2) abolishes silencing suppression, implying that suppression by this protein involves events that occur in the nucleus (13). In contrast, we have shown that AL2 exists in both the nuclear and cytoplasmic compartments in TGMV-

infected cells, providing an opportunity for interaction with ADK, which is believed to be a cytoplasmic protein (64). It is possible that some of the mutations introduced in the *Tomato yellow leaf curl virus-China* study affected amino acid residues required both for suppression and nuclear localization or interfered with ADK interaction, or perhaps rendered the C2 protein inactive. It is also possible that AL2 proteins suppress silencing through multiple mechanisms by impacting steps that occur in the cytoplasm or the nucleus.

A comparison of viral suppressor protein phenotypes is informative. In the system employed here, GFP mRNA accumulation was highest with p19, whereas the geminivirus proteins were weaker suppressors that allowed much less GFP mRNA to accumulate even though they effectively reduced siRNA accumulation over a 5-day period. There is sound evidence that p19 acts by binding and sequestering siRNAs (10, 29, 46). Thus, the disparity in suppression efficiency, coupled with our observation that AL2/L2 proteins do not bind siRNA, argues that the geminivirus and tombusvirus proteins target different steps in the silencing pathway. HC-Pro also supported more robust GFP mRNA accumulation than AL2 and L2, but unlike the geminivirus proteins, it did not prevent the accumulation of GFP-derived siRNAs through 5 days postinfiltration and did not cause a significant reduction in ADK activity in infiltrated tissue. Thus, the mechanism by which AL2 and L2 suppress silencing appears to be different from those of HC-Pro and p19.

Various transient silencing protocols sometimes yield different outcomes with the same suppressor proteins. This is particularly true for siRNA accumulation, where variability may be attributed to the time points examined (days postinfiltration), the presence or absence of a strong silencing inducer (e.g., a dsRNA-generating construct), and whether the silenced locus is present on a transient template or is a transgene (18, 25, 35, 36, 46, 56). Nevertheless, it is interesting that we and others have observed p19 and HC-Pro to differentially affect siRNA accumulation given recent evidence indicating that both proteins can block siRNA/siRNA* duplex unwinding (10, 29). One interpretation of p19's ability to reduce siRNA accumulation, originally proposed by Silhavy and colleagues, suggests that the p19-siRNA interaction additionally interferes with an amplification step mediated by siRNA and RNA-dependent RNA polymerase (23, 34, 46, 54). We speculate that the ability of AL2 and L2 proteins to reduce siRNA accumulation also results from interference with an amplification step but one that requires ADK activity and does not involve sequestration of siRNA.

Our data demonstrating that ADK activity is required for RNA silencing in intact leaf tissues appear to explain previous observations that transgenic plants with reduced ADK activity due to silencing revert at high frequency (39, 64). Further, with the revelation that this nucleoside kinase activity is important for silencing, ADK inactivation by the geminivirus pathogenicity factors AL2 and L2 may now be viewed as a counterdefense against this antiviral pathway. However, this does not contradict our previous hypothesis that ADK inactivation contributes to the suppression of SNF1-mediated responses (21, 64). It would be interesting if, by targeting a single metabolic enzyme, geminivirus AL2 proteins managed to interfere with two distinct antiviral defenses.

Why is ADK required for RNA silencing? It seems most likely that this housekeeping enzyme plays an indirect, supporting role in the process. Beginning from this premise, one possible answer to the question is that the energy requirements of the silencing pathway demand efficient, ADK-mediated adenosine salvage. Perhaps a more attractive answer arises from recent evidence showing that ADK plays a critical role in sustaining *S*-adenosylmethionine-dependent methyltransferase activity and that ADK-deficient yeast and *Arabidopsis* exhibit reduced methylation (30, 39, 45, 66). This suggests a link with viral pathogenesis because DNA and specific types of histone methylation (e.g., H3K9) are known to be associated with RNA silencing and other, predominantly negative, epigenetic pathways (for reviews see references 3, 4, and 40). Methylation would be important in an *Agrobacterium*-based transient system like the one employed in our studies if T-DNA templates were subject to epigenetic modification. If this is the case, then the recent observation that TGMV AL2 protein cannot suppress silencing directed against a GFP reporter gene in a protoplast transfection assay might reflect differences in system requirements and differences in the structures of plasmid and T-DNA-derived templates (42). T-DNA templates may well be associated with chromatin and could possibly be methylated. Plasmid-borne silencing loci may be inaccessible to methyltransferase activities, and thus methylation (and ADK activity) may not be a significant factor in protoplasts.

That plants might use methylation as a defense implies that the geminivirus minichromosome is also a target for DNA and/or histone methyltransferases. In support of this idea, we previously found that in vitro methylation of TGMV DNA impairs its ability to replicate in tobacco protoplasts. However, we also found that progeny viral DNA, and DNA isolated from *N. benthamiana* plants infected with wild-type TGMV, was not densely methylated (7). A more thorough examination of the methylation state of the geminivirus minichromosome in inoculated tissues from host and nonhost plants is ongoing, as are studies to investigate the role of ADK activity in RNA silencing.

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