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PII: S0378-1119(18)30866-7
DOI: doi:[10.1016/j.gene.2018.08.009](https://doi.org/10.1016/j.gene.2018.08.009)
Reference: GENE 43128
To appear in: *Gene*
Received date: 17 February 2018
Revised date: 12 June 2018
Accepted date: 3 August 2018

Please cite this article as: T. Vinutha, Gaurav Kumar, Varsha Garg, Tomas Canto, Peter Palukaitis, S.V. Ramesh, Shelly Praveen , Tomato geminivirus encoded RNAi suppressor protein, AC4 interacts with host AGO4 and precludes viral DNA methylation. *Gene* (2018), doi:[10.1016/j.gene.2018.08.009](https://doi.org/10.1016/j.gene.2018.08.009)

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Tomato geminivirus encoded RNAi suppressor protein, AC4 interacts with host AGO4 and precludes viral DNA methylation

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ABSTRACT

Plant RNA silencing systems are organized as a network, regulating plant developmental pathways and restraining invading viruses, by sharing cellular components with overlapping functions. Host regulatory networks operate either at the transcriptional level via RNA-directed DNA methylation, or at the post-transcriptional stage interfering with mRNA to restrict viral infection. However, viral-derived proteins, including suppressors of RNA silencing, favour virus establishment, and also affect plant developmental processes. In this investigation, we report that Tomato leaf curl New Delhi virus-derived AC4 protein suppresses RNA silencing activity and mutational analysis of AC4 showed that Asn-50 in the SKNT-51 motif, in the C-terminal region, is a critical determinant of its RNA silencing suppressor activity. AC4 showed interaction with host AGO4 but not with AGO1, aggregated around the nucleus and influenced cytosine methylation of the viral genome. The possible molecular mechanism by which AC4 interferes in the RNA silencing network, helps virus establishment, and affects plant development is discussed.

Key words: DNA methylation; RNA-dependent DNA methylation (RdDM); RNA silencing; Tomato leaf curl New Delhi virus (ToLCNDV); Transcriptional gene silencing; Viral suppressor of RNA silencing (VSR)

1. Background

RNA silencing is a conserved phenomenon in eukaryotes that is involved in gene regulation, maintenance of chromatin stability, and acts as an adaptive defence mechanism against invading pathogens, including pathogenic viruses, thus influencing overall growth and development of an organism (Martinez et al., 2013). In general, the biochemical, molecular factors, and set of genetic pathways of RNA silencing have been largely deciphered based on the investigations from plant-virus interactions (Ding, 2010). Overlapping pathways of RNA-mediated gene silencing involve active interplay of host proteins such as DNA-dependent RNA polymerases IV, members of a Dicer-like (DCL) class of ribonuclease, RNA-dependent RNA polymerases (RDRs) and RNA-induced gene silencing complexes (RISC) with slicer protein called argonautes (AGO) (Bernstein et al., 2001; Dalmay et al., 2000).

Plant-infecting viruses are inducers and targets of RNA silencing (Baulcombe 1996). Upon virus infection, double-stranded RNA (dsRNA) molecules of viral genomic origin are processed into 21- or 22-nt small interfering RNAs (siRNAs) due to the activity of DCL2 and DCL4 (Elbashir et al., 2001). In cytoplasm, these resultant siRNAs target homologous viral genomic and/or messenger RNAs and cleave cognate RNA with the aid of RISC, in the process called post-transcriptional gene silencing (PTGS) (Hamilton and Baulcombe 1999; Baulcombe, 2004). On the other hand, in the nucleus, 24-nt siRNAs derived from dsRNA, processed by DCL3, are recruited onto RNA induced transcriptional silencing (RITS) complex to cause transcriptional gene silencing (TGS). Besides AGO4, the RITS also contains other proteins such as the methyltransferases DRM1, DRM2, methyltransferase 1 (MET1) and chromomethylase 3 (CMT3) that are essential for methylating DNA of viral origin and effectively repress viral

replication (Hamilton et al., 2002; Qi et al., 2005; Xie et al., 2004; Zilberman et al., 2004). RNA silencing also encompasses amplification and spread of silencing signals wherein host RDR play a considerable role (Kalantidis et al., 2008).

Plants regulate their gene expression through DNA methylation of cytosine nucleotides. *De novo* methylation is a component of the RNA silencing pathway operating through RNA-directed DNA methylation (RdDM). Thus, RdDM activity overlaps with the host gene silencing system, which regulates gene expression and defends against invading nucleic acids such as transposons, retrotransposons and viruses, and transgenes (Pooggin, 2013; Yang et al., 2013). As a counter defence, viral proteins interfere with the plant's methylation machinery to enhance the virus accumulation (Raja et al., 2008). The TGS against DNA viruses, such as members of the family *Geminiviridae*, was shown by the following observations: (a) geminivirus DNA is methylated during infection (Hagen et al., 2008; Rodriguez-Negrete et al., 2009; Paprotka et al., 2011); (b) hypermethylation of viral DNA results in host recovery (Raja et al., 2008); (c) viral genome methylation affects the replication of geminiviruses in tobacco protoplasts, as shown for Tomato golden mosaic virus (Brough et al., 1992) and African cassava mosaic virus (Ermark et al., 1993); (d) components of the methylation machinery, such as histone 3 lysine 9 (H3K9) methyltransferase and adenosine kinase (ADK), are involved in viral DNA methylation and confer resistance against infection (Raja et al., 2008); and e) two geminivirus [Tomato golden mosaic virus (TGMV) and Cabbage leaf curl virus (CaLCuV)]-derived AC2 encoded Transcriptional activator protein (TrAP) targets histone methyltransferase, and Su(var)3-9 homolog 4/Kryptonite (SUVH4/KYP) proteins to attenuate the host TGS (Castillo-González et al., 2015). These studies indicate elicitation of TGS during geminivirus invasion is a major defence

mechanism that is counteracted by the viral genome-encoded proteins. Reports also suggest that the geminiviral-encoded C2 protein can reverse TGS by interfering with the production of S-adenosyl methionine, an essential methyltransferase cofactor (Buchmann et al., 2009; Zhang et al., 2011). Also, C2 suppresses TGS through inhibition of ADK (Wang et al., 2003) and/or attenuation of proteasome-mediated degradation of S-adenosyl-methionine decarboxylase 1 (Zhang et al., 2011). Geminivirus replication associated protein (Rep; also known as C1 or AC1 or AL1), down-regulates the expression of MET1 and CMT3 (the key components of the methylation machinery), whereas the C4 protein plays an indirect role in the suppression of MET1 (Rodriguez-Negrete et al., 2013). The geminivirus beta satellite DNA-encoded protein, β C1, interferes in the methylation cycle by inhibiting the activity of S-adenosyl homocysteine hydrolase (Yang et al., 2011).

Tomato leaf curl New Delhi virus (ToLCNDV) is a bipartite, whitefly-transmitted geminivirus that causes devastating leaf curl disease of tomato, potato and cucurbits grown in northern India. Infected tomato plants exhibit severe stunting, leaf curling, interveinal yellowing, and yield levels are drastically reduced depending on the stage of virus infection (Chakraborty, 2008). Hence, the RNA silencing phenomenon has been exploited to develop antiviral resistance in tomato. Previously, we reported that constitutive expression of intron-spliced hairpin RNA targeting Rep protein, encoded by open reading frame (ORF) AC1 of ToLCNDV, is a potent strategy to obtain transgenic resistance in tomato (Ramesh et al., 2007). Furthermore, ectopic expression of short hairpin RNA directed against ToLCNDV derived AC4 mRNA resulted in resistance to leaf curl disease concomitant with the development of phenotypic abnormalities in tomato (Praveen et al., 2010). Geminiviruses encode multiple viral suppressors of RNA silencing (VSR) *viz.*,

AC2/C2 encoded TrAP (Wang et al., 2003; Yang et al., 2007; Zhang et al., 2011; Yang et al., 2013), AC4 protein (Vanitharani et al., 2004) and V2 protein (Luna et al., 2017). However, VSRs encoded by ToLCNDV have not been investigated in depth to gain molecular insights regarding the overlapping gene silencing pathways that control overall plant growth and development and that confers virus resistance. Hence, as a follow-up to the observation of phenotypic abnormalities in RNA silencing-based tomato transgenics, we investigated the mechanism underlying the potential VSR activity of ToLCNDV-derived AC4. Molecular evidence from this work substantiates that AC4 interacts with host AGO4. While expression of AC4 prevents viral genome methylation, sub-cellular localization studies reveal that AC4 was found to be distributed in the cytoplasm, aggregated around the nucleus and displayed little *in vitro* small RNA (sRNA) binding capability. Hence, it is proposed that the ToLCNDV encoded AC4 suppresses RNA silencing both at TGS and PTGS levels, which affects normal developmental processes of the tomato plants.

2. Methods

2.1. Plant materials, growth conditions and ToLCNDV inoculation

Tomato (*Solanum lycopersicum* var. Pusa Ruby) plants were grown at the National Phytotron Facility, ICAR-IARI, New Delhi and were maintained under 16/8 h light/dark periods, 18,000 lx, 28–30 °C, and 85 % relative humidity. Healthy tomato Pusa Ruby (PRu) plants along with transgenic tomato lines (PRu AC4-sh, PRu AC4-IR and PRu AC4-IHP) developed in our laboratory (Ramesh *et al.*, 2007; Praveen et al., 2010) with cassettes containing CaMV 35S promoter-driven short-hairpin (PRu AC4-sh), inverted repeats (PRu AC4-IR), or intron-spliced hairpin RNA (PRu AC4-IHP), targeting AC4 mRNA, were used. Both the non-transformed Pusa Ruby (NT-PRu) and transgenic tomato (PRu-AC4, PRu AC4-sh, PRu AC4-IR

and PRu AC4-IHP) plants were inoculated with ToLCNDV following the established agroinoculation protocol (Jyothsna et al., 2013). The relative abundance of AC4 mRNA levels was quantified in these transgenic plants and normalized with reference to the endogenous control actin. Oligonucleotides for PCR amplification used in this study are presented in Table 1. Also, viral gene accumulation (fold change with respect to the susceptible genotype) was studied 10, 20 and 30 days after agroinoculation. In these studies, five biological replicates of all the tomato genotypes were used and the experiment was repeated twice.

2.2. DNA bisulfite sequencing

Total DNA was isolated from NT-PRu and both PRu-AC4 and PRu-AC4-IHP plants 30 days after infection with ToLCNDV using the DNeasy plant mini kit (Qiagen, Clifton, Australia). Bisulfite conversion of DNA was carried out using EpiTech Bisulfite kit (Qiagen). High resolution melt analysis was accomplished to monitor the bisulfite conversion rate. To determine the methylation status of the AC4 gene, a 396-bp GC rich region encompassing the complete AC4 gene and a flanking sequence (conserved *Rep* gene) was selected. Sequence-specific primers were designed using Meth software version 5.0 to amplify GC rich region of the AC4 gene from bisulfite-converted DNA and the primers are given in Table 1. PCR products were resolved in a 1% agarose gel, and gel purified using the QIAquick gel extraction kit (Qiagen). PCR amplicons were ligated into pGEM-T-Easy (Promega), and introduced into *E. coli* DH5 α cells according to the manufacturer's instructions. Recombinant plasmid DNA (10 independent clones for each condition) was extracted from *E. coli* using a Qiagen Spin miniprep kit (QIAGEN) and sequenced with T7 or SP6 primers (Chromous Biotech, India). Sequence information was analyzed for the methylation using BioEdit Sequence Alignment Editor Version

5.0.9 (Hall, 1999). One-way analysis of variance (ANOVA) was done to compare the mean values and differential methylation pattern was analyzed following T-test at significance levels of $P < 0.01$.

2.3. Construction of plasmids

The ToLCNDV-derived AC4 gene (GenBank HQ141673) was manipulated to generate various plasmids following standard molecular biology protocols (Sambrook and Russell, 2001). To develop gene constructs, the target viral genome sequence of ORF AC4 [177 bp (1-177 of AC4)] was selected, PCR amplified and cloned in the pGEMT-Easy vector. The insert was sub-cloned under the transcriptional control of the CaMV35S promoter and 35S terminator in the vector pUC118 to develop the recombinant pUC-118-AC4 vector. Using pUC118-AC4, the gene construct was later sub-cloned in pCAMBIA 2301 using restriction enzymes *EcoRI* and *Hind III* to obtain pCAMBIA 2301(AC4). *Agrobacterium tumefaciens* strain LBA4404 was transformed with binary plasmid pCAMBIA 2301(AC4) following the freeze thaw method (Chen et al. 1994).

2.4. Yeast Two-Hybrid (Y2H) and Bimolecular fluorescence complementation (BiFC) assay

The full-length coding sequences of AC4 and its mutants were PCR amplified using the primers AC4-*EcoRI* F and AC4-*BamHI* R. The PCR products were subjected to restriction digestion using enzymes *EcoRI* and *BamHI* followed by ligation and cloning in the yeast vectors pGADT7 (AD) and pGBKT7 (BD). To determine the interaction of different pairs of proteins, individual protein-coding genes were ligated in the pGADT7 (AD) and pGBKT7 (BD) vectors and were co-transformed in *Saccharomyces cerevisiae* (strain AH109) using the EZ-Yeast transformation kit (MP Biomedicals). Transformants grown on auxotrophic double

dropout media (SD/-leu/-trp) were confirmed by colony PCR using the respective primers. The co-transformed colonies from double dropout plates were streaked on quadruple dropout agar media (SD/-leu/-trp/-ade/-his+x-alpha-gal) as described in the yeast protocol handbook (ClonTech). To confirm the expression of genes of interest, the expressed proteins were detected by western blotting using anti-c-myc antibodies with a dilution of 1:1000 in PBS (ClonTech) as described in the Yeast Protocol Handbook (ClonTech).

For BiFC studies, *Agrobacterium* cultures, each carrying a binary vector with the corresponding split yellow fluorescent protein NorC-termini (sYFPN- or sYFPC-) fused to the proteins of interest (AGO4), were co-infiltrated together into the same leaf tissue. Epidermal cells of *N. benthamiana*-infiltrated tissue were monitored for fluorescence from either green fluorescent proteins (GFP), or sYFP fluorophores of tagged proteins. Imaging was made with Leica SP1 and SP2 (Leica Microsystems, Heidelberg, Germany) confocal laser scanning microscopes, using fresh, non-treated leaf tissues as previously described (Canto et al. 2006) .

2.5. Protein extraction and Western blot analysis

To extract total protein from plant samples, about 100 mg of infiltrated leaf tissues were ground to a fine powder in pestle and mortar using liquid nitrogen. Total plant proteins were extracted using QB buffer (Protocols, The Stockinger Lab) and quantified (Bradford, 1976). An equal volume of 2x Laemmli loading buffer (pH 6.8 comprising 1% 2-mercaptoethanol, and 1% SDS) was added before boiling, which occurred for 5 minutes, and protein fractionation was carried out in 12% SDS-PAGE.

Total yeast protein was extracted following the method described by Kushnirov (2000). Briefly, 3ml of overnight grown yeast culture was centrifuged at

10000 rpm at 4 °C and the resultant pellet was washed with sterilized distilled water (SDW) and was resuspended in equal volumes (200 µl) of SDW and 0.5 N NaOH, and incubated at room temperature for 10 min. This was followed by centrifugation to harvest the pellet of cells that was dissolved in 100 µl of SDS sample buffer (60 mM Tris-HCl, 2%SDS, 5% glycerol, 4% β-mercaptoethanol and 0.0025 % bromophenol blue). The sample was then boiled for 5 min, centrifuged, and the supernatant was used for loading and fractionation by SDS-PAGE.

Proteins in the gel were wet-blotted onto nitrocellulose membranes. For immunological detection of GFP, a goat anti-GFP polyclonal antiserum (Sigma) was used. Blotted proteins were detected using commercially available alkaline phosphatase conjugated anti-goat secondary antibodies (Sigma) and Amresco Fast BICP/NBT substrate. GFP antibodies, with a dilution of 1:500 in PBS (G-Biosciences) were used to detect GFP in the agropatch assay.

2.6. *In vitro* sRNA binding and gel-shift mobility assays

Double-stranded miRNA171 (miR171 and its complement) was produced by mixing equimolar amounts of chemically synthesized small single-stranded RNAs (miRNA 171 and miRNARc), followed by boiling and cooling to room temperature. For *in vitro* protein–RNA binding assays, 30 pmol of synthetic miRNAs were incubated with different concentrations of protein(s) under a temperature gradient in a buffer comprising 10 mM Tris/HCl among others as enumerated by Rakitina et al., 2006. Protein-RNA complexes were analyzed in 2% agarose gels in Tris-acetate buffer as described by Rakitina et al. (2006). RNA bands were visualized by ethidium bromide staining and quantified using an Alpha Imager version 6.0.

For electrophoretic mobility shift assays, the native and mutant AC4 were expressed in bacterial systems using pMAL vector (pMAL protein expression and

purification system, NEB). Briefly, recombinant pMAL plasmids were developed with wild-type AC4 and mutant *AC4* genes as inserts using the primers AC4-*EcoRI* F and AC4-*Pst* I R (Table 1). Bacterial expression and purification of AC4 and mutant proteins were done as described by Rodriguez and Carrasco, (1993) with minor modifications. *E. coli* (strain BL21) cells were transformed with pMAL-C2X encoding MBP- α galactosidase-peptide fusion and pMAL-AC4/M1/M2/M3 with a *tac* promoter to express MBP-AC4 fusion protein (Mangrauthia et al., 2009). The fusion proteins were affinity-purified by chromatography and the purified fusion proteins were quantified using a NanoDrop 2000, UV-Vis, Thermo Scientific™ and then separated by 12% SDS-PAGE.

2.7. Construction of *AC4* mutants

To assess the RNA silencing suppressor activity of the ToLCNDV-derived *AC4* gene in transient assays, wild-type and mutant forms of the *AC4* gene were generated and cloned in the binary plasmid pCAMBIA2301. *AC4* mutants, viz., AC4A³⁰V, AC4N⁵⁰H and AC4M⁵⁵T, were generated following PCR-mediated site-directed mutagenesis. Mutations in *AC4* gene were confirmed through Sanger sequencing. PCR amplification of complete *AC4* gene and mutants were performed using the primers AC4-*Apa*IF and AC4-*Xho*I R. The amplicons were then digested with restriction enzymes *Apa*I and *Xho*I followed by directional cloning in vector pUC118. The recombinant pUC118 plasmids were restricted with enzymes *Eco*RI and *Hind* III to release the gene inserts flanked with CaMV 35S promoter and CaMV 35S terminator sequences followed by sub-cloning in binary plasmid pCAMBIA2301.

2.8. 3D protein structure prediction

The 3D structures of AC4 and mutants AC4A³⁰V, AC4N⁵⁰H and AC4M⁵⁵T were predicted using the Phyre server, with domain analysis and multiple template *ab initio* modelling (Kelley and Sternberg, 2009).

2.9. Intracellular localization studies

In the sub-cellular localization studies, AC4 and its mutant were cloned into the vector pROK2-GFP (González et al., 2010) to generate GFP-AC4 and GFP-AC4A³⁰L, GFP-AC4N⁵⁰H and GFP-AC4M⁵⁵T plasmids respectively. *Agrobacterium* (strain C-58) cultures, each carrying a binary vector harbouring GFP fused to the proteins of interest, were infiltrated into leaf tissues of *N. benthamiana*. Epidermal cells of infiltrated tissue were monitored for fluorescence. Imaging analysis was performed with LeicaSP1 and SP2 (Leica Microsystems) confocal laser scanning microscope, using fresh, non-treated leaf tissue and water immersion or water-dipping objectives (Canto et al., 2006).

2.10. Transient or agropatch assay

Chemically competent cells of *A. tumefaciens* strain LBA4404 were transformed with recombinant binary vectors harbouring full length AC4 and mutant genes. Transient expression assays (agropatch assays), were performed through infiltration of the respective *A. tumefaciens* cultures. Briefly, single colony cells of *A. tumefaciens* were grown overnight at 28 °C and prior to infiltration each bacterial culture was diluted to final OD of 0.2 at 600nm. *A. tumefaciens* cultures harboring various plasmids were then combined and leaves of *N. benthamiana* lines were infiltrated following the protocol suggested by Canto and Palukaitis (2002).

3. RESULTS

3.1. Differential accumulation of AC4 mRNA in transgenic tomato

Various gene construct strategies such as short hairpin RNA (AC4-sh), inverted repeats (IR) targeting AC4 (AC4-IR) and intron-spliced hairpin (IHP) RNA (AC4-IHP) were developed to downregulate the viral AC4 gene expression in transgenic tomato lines (Ramesh et al., 2007; Praveen et al., 2010). Transgenic tomato lines were challenge inoculated with ToLCNDV (Fig. 1A) and relative expression levels of AC4 transcripts were examined 10 days post inoculation to determine the gene silencing efficiency of the various constructs (Fig. 1B). As expected, AC4 mRNA level was found to be high in transgenic PRu-AC4 (over-expressing AC4). In tomato expressing AC4 shRNA (PRu-AC4-sh), AC4-IR (PRu-AC4-IR) and AC4-IHP (PRu-AC4-IHP) the levels of AC4 mRNA were reduced than those in PRu-AC4 plants. Thus, differential degrees of RNA silencing of AC4 mRNA were observed with the expression of various gene constructs (Fig. 1B).

3.2. AC4 affects cytosine methylation of viral DNA

Methylation of viral genomic DNA was ascertained after agroinoculation with ToLCNDV in three different tomato genetic backgrounds: (i) non-transformed (NT) PRu tomato, (ii) PRu-AC4 tomato, and iii) PRu-AC4-IHP tomato (Fig. 1A). Prior to methylation studies, relative accumulation of viral DNA was analyzed in all three genotypes at three time points i.e., 10, 20, and 30 days post agroinoculation (Fig. 1C). All three genotypes exhibited a progressive increase in viral DNA accumulation with time, even though the absolute quantity of viral DNA accumulation was found to vary with the genetic background. Since the cultivar Pusa Ruby (PRu) is susceptible to ToLCNDV infection, viral titre was found to be increasing with time even at 30 dpi. At any given point of time, NT PRu tomato

showed relatively high viral DNA levels, followed by PRu-AC4 plants, whereas the least amount of viral DNA was found in PRu AC4-IHP plants. This implies that constitutive expression of siRNAs generated from IHP RNA effectively targets AC4 mRNA so that viral titer levels were lower in PRu-AC4-IHP tomato plants (Fig. 1C).

Methylation levels of select viral genomic DNA were examined to determine if RdDM acts as a defence mechanism in ToLCNDV-infected transgenic PRu plants [(PRu AC4) and (PRu AC4-IHP)] and in control (NT-PRu) plants. Similarly, any molecular link between the process of viral DNA methylation and RNA silencing of *AC4* gene due to the expression of AC4-IHP in PRu AC4-IHP plants was also investigated. The selected viral genomic region (of 396 bp) was within AC1 ORF (nucleotide positions 2113-2509). It encompasses the full length AC4 ORF (2251-2427) and a conserved *Rep* region (nucleotide positions 2365-2621). A total of 94 cytosine residues are present in the selected viral genomic region, out of which 27 are symmetric cytosines (CG, CNG where N is A, T, C, or G) and 67 are asymmetric cytosines (CHH residues where H is A, T or C). Bisulfite sequencing revealed a higher level of cytosine methylation (69.1%) in PRu AC4-IHP plants than in NT-PRu plants (50%). In addition, 53 and 28 asymmetric cytosine bases were methylated, respectively, in PRu AC4-IHP plants (~79.1% of total asymmetric cytosines) vs. NT-PRu plants (~42% of total asymmetric cytosines) (Fig.1D). However, no significant difference in methylation level of symmetric cytosines was observed in PRu AC4-IHP plants (46% of symmetric cytosines) vs. NT-PRu plants (53.8% of symmetric cytosines), where 12 and 14 cytosines were methylated, respectively (Fig.1D).

3.3. *ToLCNDV-AC4 interacts with host AGO proteins but not with sRNAs*

ToLCNDV-derived AC4 affected cytosine methylation, hence, the possibility of an interaction between AC4 and the host-derived AGO4 protein, an important component of DNA methylation machinery, was assessed. Yeast two-hybrid assay revealed that host AGO4 and ToLCNDV derived AC4 showed protein: protein interaction regardless of whether the AC4 and AGO4 were fused to the activation or binding domains. On the other hand, AC4 did not show any interaction with AGO1, that is mainly involved in PTGS of plants (Fig. 2Ai). The interaction between AC4 and AGO4 was further confirmed in BiFC studies using split YFP fusions of AC4 protein, which was co-expressed with AGO4, by agro-infiltration of *N. benthamiana* leaves (Fig. 2Aii). Interactions of AC4 and AGO4 proteins were detected by BiFC in both the orientations ie) proteins were either tagged in N or C-termini (Fig. 2Aii). The expression of ToLCNDV derived AC4 and host AGO1 and AGO4 proteins were confirmed through western blotting by detecting C-myc in BD vector (Fig. 2B).

Purified native AC4 protein (Fig. 2Ci) was assessed for sRNA binding abilities. Gel mobility shift assays reveal that native AC4 do not show any affinity for sRNAs *in vitro*. Varying concentrations of purified AC4 protein (up to 500 pmol) were incubated with 30 pmol of synthetic, small dsRNAs (siRNA171) at constant temperature and time showed little interaction (Fig. 2Cii). Similarly, when a constant concentration of AC4 protein (500 p mol) was incubated with 30 pmol of small dsRNAs for varying time periods, no shift in the mobility of AC4 was observed (Fig. 2Ciii). It indicates that ToLCNDV encoded AC4 protein may not utilize sRNA binding as a fundamental strategy to suppress host induced RNA

silencing. Thus, these observations suggest that AC4 might indirectly influence the sRNA homeostasis in the cell.

3.4. Sequence analysis of AC4 protein

A multiple sequence alignment of amino acid sequences of AC4 protein obtained from 23 variants of ToLCNDV infecting tomato and other solanaceous crops was generated (Fig. 3A). Three amino acids (Ala at position 30, Asn at position 50 and Met at position 55) were found to be either unique or occurred infrequently in ToLCNDV variants. Hence site-directed mutagenesis was used to generate three different AC4 mutants (AC4A³⁰V, AC4N⁵⁰H and AC4M⁵⁵T) (Fig. 3B). These mutants, along with native AC4, were used in subsequent studies to evaluate whether these three specific amino acids affected important functions.

To ascertain the effect of mutational changes in protein structure and function, wild-type AC4 and the three mutants were analyzed for their potential 3D structure. Mutant AC4N⁵⁰H displayed an altered 3D structure, having an extra β sheet (Fig. 3Ci-ii), which resulted in the formation of a hydrophilic pocket/domain. Ramachandran plot analysis (RAMPAGE analysis) of AC4N⁵⁰H protein revealed that two amino acids (D²² and R⁴⁰) residues were present in non-permissible locations. These disturbances were observed in the Src Homology 3 Domain-like Fold (SH3-domain-like fold) of AC4N⁵⁰H but not in AC4A³⁰V and AC4M⁵⁵T.

3.5. Cytosolic protein AC4 is a VSR and Asn-50 is crucial for this activity

To investigate the sub-cellular localization of the AC4 protein, native AC4 and the three mutants were fused with the green fluorescent protein (GFP) to produce GFP tagged proteins. These proteins were constitutively expressed from binary vectors (35S-GFP-AC4, 35S-GFP-AC4A³⁰L, 35S-GFP-AC4N⁵⁰H and 35S-GFP-AC4M⁵⁵T) in *N. benthamiana* plants. After agro-infiltration of *N. benthamiana*

leaves followed by confocal microscopy visualization, these proteins were found to be in the cytoplasm and aggregating around the nucleus (Fig. 4A). The subcellular localization of AC4 protein and its mutants were found to be more or less similar, except lower aggregation of GFP-AC4N⁵⁰H around the nucleus was observed.

The potential RNAi suppression activity of the ToLCNDV-encoded AC4 protein and the three mutants derived from it were evaluated in leaf infiltration assays by co-expression with 35S-GFP in the leaves of *N. benthamiana* plants. Leaves infiltrated with 35S-GFP and the empty binary vector did not display GFP fluorescence at 5 dpi, as a consequence of RNA silencing activation (Fig. 4B). By contrast, leaves co-infiltrated with *Agrobacterium* harbouring 35S-GFP and a 35S-driven gene encoding the Tomato bushy stunt virus VSR, P19, showed intense GFP fluorescence, indicating that RNA silencing was suppressed. Among the experimental plants infiltrated with *Agrobacterium* harbouring the native and the three mutated AC4 genes, bright green fluorescence was maintained at 5 dpi in leaves co-infiltrated with 35S-GFP and 35S-AC4, 35S-AC4A³⁰V and 35S-AC4M⁵⁵T. Interestingly, leaves infiltrated with 35S-GFP together with 35S-AC4N⁵⁰H did show negligible green fluorescence (Fig. 4B). This experiment demonstrated that the ToLCNDV-AC4 and two of the mutants, excluding mutant AC4N⁵⁰H, are strong VSRs.

Expression level of GFP was high in leaves co-infiltrated with the GFP construct and AC4 or the P19 construct than in leaves co-infiltrated with the GFP construct and the empty vector (Fig. 4C). Levels of GFP expression in leaves that were co-infiltrated with AC4 mutants AC4A³⁰V and AC4M⁵⁵T also were similar to those of the positive controls; however, the mutant AC4N⁵⁰H showed very low GFP expression at 5 dpi, similar to the negative control (Fig. 4C).

The three AC4 mutants AC4A³⁰V, AC4N⁵⁰H and AC4M⁵⁵T were expressed in yeast shuttle vectors. In the yeast two-hybrid assay, interaction of these proteins with the tomato AGO4 was observed, regardless of the orientation of interacting proteins (Fig. 4D). Western blot analysis did not reveal any differences in the expression levels of the mutants and wild-type proteins (Fig. 4E).

Altogether, the results of the agropatch VSR assay, Western blotting and 3D structure analysis of AC4 and the three mutants suggest a possible novel function of the Asn at position 50 in regulating RNA silencing suppression activity. Thus, it appears that distorted 3D structure of AC4 protein resulting from this mutation (AC4N⁵⁰H) could potentially have affected the RNA silencing suppression activity of AC4.

4. DISCUSSION

Geminivirus-derived proteins, mostly VSRs target both PTGS and TGS pathways by binding sRNAs and modulating enzymes involved in the host methyl cycle, respectively. Furthermore, they interact at multiple steps of the RNA silencing pathway, thereby debilitating the host defence mechanism (Anandalakshmi et al., 1998; Silhavy et al., 2002; Csorba et al., 2007; Giner et al., 2010). Viral derived-transcriptional activator protein (TrAP) or its homologs AC2/C2 exhibit RNA silencing suppressor activity by inhibiting or degrading host enzymes (Yang et al. 2007; Chellapan et al., 2005; Zhang et al., 2011; Castillo-González et al., 2015; Sun et al., 2015; Kumar et al., 2015). Alternatively, AC2 (of Cabbage leaf curl virus) and its monopartite homolog C2 (of Beet curly top virus) reverse the RNA silencing in a manner independent of transcriptional activation suggesting multiple layers of viral counter-defence (Jackel et al., 2015). Similarly, geminivirus-derived V2 also functions as a strong suppressor of RNA silencing by

weakening RDR-6/suppressor of gene silencing-3 (SGS-3) pathway (Luna et al., 2017) suggesting the multi-dimensional role of geminivirus-derived proteins.

TrAP or AC2, appears to be the major VSR of a geminivirus family however it does not preclude a VSR role for AC4 (Vanitharani et al., 2014; Chellappan et al., 2005). Since, methylation of the viral genome is an important antiviral mechanism of plants (Bian et al., 2006; Raja et al., 2008) the effect of host-induced methylation of ToLCNDV genomic DNA was examined while the viral AC4 gene was silenced (PRu-AC4-IHP) and over-expressed (PRu-AC4). Constitutive expression of *AC4* gene reduced the intensity of host-induced methylation of viral genome implying a role for AC4 protein in modulating the host methylation machinery. Similarly, begomovirus-derived proteins (Tomato golden mosaic virus-encoded AL2 and Beet curly top virus-encoded L2) severely reduce global cytosine methylation by inhibiting host adenosine kinase (ADK), involved in the maintenance of the methyl cycle, thereby negating the effect of TGS (Wang et al., 2005). AC4 could potentially interfere with the host methylation machinery to protect the viral genomic regions from the effects of methylation. In plants AGO4 is involved in methylation of invading viral nucleic acids by recruiting 24-nt viral siRNAs (Mallory et al., 2010). VSRs also arrest the formation of functional RISC assembly by interacting with host AGO proteins. Our data show AC4 interacts with the tomato AGO4 but not with AGO1 further corroborating AC4's role in altering the host methyl cycle to favour ToLCNDV.

A widespread strategy employed by plant virus-derived VSRs is sequestration of small dsRNA, to prevent RISC assembly or RISC loading, (Lakatos et al. 2006; Zhang et al., 2012). ToLCNDV-derived AC4 does not bind small dsRNA indicating it could potentially employ a different molecular strategy other

than sRNA binding to reverse the RNA silencing. However, the V2 protein of Tomato yellow leaf curl China virus (TYLCCV) binds 21 nt and 24 nt siRNA duplexes, inhibits local and systemic RNA silencing even though it does not interact with SGS3-a dsRNA binding protein of RNA silencing pathway (Zhang et al., 2012).

ToLCNDV encoded AC4 interacted with host AGO4 and concomitantly lacked sRNA binding ability, suggesting it might play a role in the reversal of the PTGS mechanism. Agro-patch assay proved that wild-type AC4 and two mutants, but not AC4N⁵⁰H, are effective PTGS suppressors. To substantiate it further, confocal microscopic images demonstrated that GFP-tagged AC4 was found distributed in the cytoplasm and aggregated around the nucleus. Interestingly, the silencing suppression-minus mutant, AC4N⁵⁰H, also showed the distribution in cytoplasm with lesser accumulation around the nucleus. On the one hand, sub-cellular localization of AC4 revealed that it is a cytosolic protein and reverses RNAi however, on the other hand, AC4 interacts with host AGO4 but not with AGO1. Hence, it is likely that AC4 exerts an indirect role in suppressing the RNA silencing process. In this context, it merits exploring any conceivable nexus between the AC4 and host-derived endogenous silencing suppressors that might be exploited by the VSR to sabotage the antiviral measures of host. Interestingly, geminivirus-encoded suppressor protein β C1 upregulates the endogenous silencing suppressor *Nbrgs-CaM* gene and its Solanaceae orthologs (Li et al., 2014). The fascinating discovery of microProteins in plants is appealing to speculate that AC4 perhaps could act as a microProtein that either inactivates the target host protein or alters the function of the target protein to help the survival of virus (Bhati et al., 2018).

A candidate tomato gene or gene library screen could identify possible protein links that actually effect AC4 mediated reversal of RNAi through subversion of endogenous silencing suppressors. The ability of ToLCNDV to encode more than one silencing suppressor with different modes of action is intriguing and a detailed study is pertinent to comprehend the significance of such a strategy evolved by geminiviruses. The functional differences of suppressor proteins, and their efficiency of silencing suppression, have been attributed to the pathogenicity differences of begomoviruses (Cui and Zhou 2004). It is also relevant to investigate the silencing suppression efficiency of AC4, and AC2 to gain molecular insights.

Secondary structure of mutant AC4N⁵⁰H was profoundly distorted due to the appearance of a β -sheet in SKNT domain that could have impaired its ability to suppress RNA silencing. The distorted SH3 domain of AC4 is a critical component involved in protein-protein interactions, receptor binding and intracellular signalling events. However, the mutation in SKNT domain did not compromise AC4-AGO4 interactions. Hence, it is likely that AC4 might promote the survival of ToLCNDV within the plant cell by modulating the pathways controlled by SH3 domain containing proteins. Alternatively, AC4 might promote virus invasion by binding to receptors present in the host cells.

5. Conclusion

This investigation provides molecular evidence to support the hypothesis that the ToLCNDV-encoded AC4 might execute multiple counter-defence strategy at both the TGS and the PTGS levels of RNA silencing. At the TGS level, prevention of viral DNA methylation occurs by sequestering functional AGO4. Since AC4 does not enter the nucleus of the cell, it is important to investigate the

underlying mechanism by which AGO4 inhibition contributes to TGS suppression. The intracellular localization of AC4 and the ability to reverse GFP silencing in *N. benthamiana* indicate that it is an effective PTGS suppressor. Hence, it is pertinent to identify other missing host factors and/or viral factors that contribute to suppression of RNA silencing in ToLCNDV-tomato.

Competing interests

The authors declare that they have no competing interests

Author contributions:

Conceived and designed the experiments: SP. Performed the experiments: VT, GK, and VG. Analyzed the data: SP, TC, PP, RSV. Wrote the paper: SP, RSV, TC, and PP. All authors have read and approved the final manuscript

Acknowledgements

This work was supported by ICAR-IARI, New Delhi. We are also thankful to Council of Scientific and Industrial Research (CSIR) and Department of Biotechnology, New Delhi for providing fellowship to GK, and VG. The Next Generation BioGreen21 Program (Grant no. PJ011309032017) of the Rural Development Administration, and Grant no. NRF-2016R1D1A1B03930764 from the Korean National Research Foundation, both from the Republic of Korea to P. P. are also acknowledged. The funding sources had no involvement in the study design, data generation, analysis or generation, or the writing of the manuscript.

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Table 1. List of oligonucleotides used.

S.No.	Activities	Primer ID	Forward sequence (5'- 3')
			Reverse primer (5'-3')
1	pGEM-T cloning, GFP-labelling, confocal and Y2H studies	AC4F	GATGAATTCATGGGTCTCCGCAT ATCCAT
		AC4R <i>EcoRI/Bam</i> HI	GCTGGATCCCTAGAACGTCTCCAT CTTTGT
2	pUC118 cloning and silencing studies	AC4F	GATGGGCCCATGGGTCTCCGCAT ATCCAT
		AC4R <i>ApaI/XhoI</i>	GCTCTCGAGCTAGAACGTCTCCAT CTTTGT
3	Mutation	AC4M1F	CTTGGTTTCCCCAAGTCGGTCAGC ACATTTCC
		AC4M1R	GGAAATGTGCTGACCGACTTGGG GAAACCAAG
4	Mutation	AC4M2F	GCGTCGGACGTCAAAACATACAT CGACAAAGATG
		AC4M2R	CATCTTTGTGCGATGTATGTTTTGA CGTCCGACGC
5	Mutation	AC4M3F	CAAAAAATACATCGACAAAGACG GAGACGTTCTAG
		AC4M3R	CTAGAACGTCTCCGTCTTTGTCGA TGTATTTTTTG
6	Bisulfite	AC4 F	ATTTTTGGGGGTTAATTTTTTT

	sequencing	AC4 R	AAAAAAACACTTTCCCAATTACA
7	sRNA/mobility shift assay	MiRNA171 MiRNA171	UGAUUGAGCCGCGCCAAUAUC GAUAUUGGCGCGGCUCAAUCA
8	Gel mobility assay	AC4 F	GATGAATTCATGGGTCTCCGCAT ATCCAT
		AC4 R <i>EcoRI/PstI</i>	GCTCTGCAGCTAGAACGTCTCCAT CTTTGT

Figure Legends

Fig.1. Phenotypic expression of transgenic tomato genotypes, viral gene accumulation and methylation analysis post ToLCNDV challenge agroinoculation.

(A) Tomato phenotypes before and after 30 days of ToLCNDV challenge agroinoculation on (i) PRu Non transgenic; PR regenerated plant from cotyledon leaf; (ii) PRu-AC4, PRu transformed with binary construct in pCAMBIA 2301-ToLCNDV-AC4; (iii) PRu-AC4-IHP, PR transformed with binary construct in pCAMBIA2301 AC4-IHP construct.

(B) Relative expression levels of *AC4* gene (quantified by real-time PCR) post 10 days ToLCNDV challenge agroinoculation in transgenic tomato genotypes (PRu-AC4, PRu-AC4-sh, PRu-AC4-IR and PRu-AC4-IHP) using $2^{-\Delta\Delta C_t}$ method normalized with reference to the endogenous control actin

(C) Graphical representation of viral gene accumulation (fold change with respect to the susceptible genotype) 10, 20 and 30 days after agroinoculation. Each value on the bar graphs represents the mean of five biological replicates of all the tomato genotypes and the experiment was repeated twice.

(D) The histogram representing the percentage of methylated viral genome sequences (CG, CHG and CHH) in a 396-bp region of the viral genome. The methylation status was obtained after sequencing bisulfite-treated total DNA using specific primers designed to amplify the converted template from the selected viral region, in PR non transgenic, PR AC4 (expressing AC4) and PR AC4-IHP (silencing AC4) transgenic tomato plants 30 days after agroinoculation. Each column represents the mean $\pm$ SE of sequencing analysis of 20 cloned PCR products. Different letter corresponds to significantly different levels of methylated cytosines in percentage. ($P < 0.01$)

Fig.2. ToLCNDV AC4 and host argonaute (AGO) interactions and its small RNA binding ability.

(A) For yeast two-hybrid assays, *Saccharomyces cerevisiae* strain AH109 cells were transformed with the plasmids. (i) (a) Schematic representation of interaction of

ToLCNDV AC4 with the host argonaute proteins AGO1 and AGO4 is shown in the plate format where pGADKT7 is denoted as AD, pGBKT7 is denoted as BD, pGBKT7-p53/pGADT7-RecT (positive control) is symbolized as +, and pGBKT7-p53-/pGADT7-lamin (negative control) as -. (b) The cells were grown on SD/-Ade/-His/-Leu/-Trp (quadruple drop out) + X- α -gal media (AC4 interacts with AGO4 but not with AGO1) streaked from (c). (c) The same cells were grown on double drop out media. (ii) Bimolecular fluorescence complementation (BiFC) between ToLCNDV AC4 and host AGO4 protein tagged at their N- and C- termini, respectively, with split yellow fluorescent protein halves (sYFP) expressed transiently by agro-infiltration in *Nicotiana benthamiana* epidermal cells: (a) sYFPN-AC4 plus sYFPC-AGO 4 (b) sYFPN-AGO4 plus sYFPC-AC4

(B) Western blot analysis of yeast total protein fractionated in a 10 % SDS-PAGE and probed with mouse anti-myc antiserum to study the expression of AC4 and argonaute proteins, along with the positive control; pGBKT7-p53/pGADT7-RecT and negative control (protein isolated from non-transformed yeast cells). The lower panel represents the loading control detected by Ponceau staining.

(C) Small RNA binding ability of AC4. Electrophoretic mobility shift assay for ToLCNDV AC4. (i) Purified preparations of AC4 protein (42 kDa) on Coomassie-stained 12 % SDS-PAGE purified from *Escherichia coli* BL21 cells. (ii) Different concentrations (lane 2-8) of purified AC4 were incubated with 30 pmol of synthetic double stranded small RNA (siRNA171) for 30 min. at 25 °C. Lane 1, 30 pmol of synthetic, double-stranded small RNAs (siRNA171) without any protein added; lane 9, 500 pmol of AC4 without any synthetic, double-stranded small RNAs (siRNA171) as control. (iii) 500 pmol of AC4 was incubated with 30 pmol of double-stranded small RNAs (siRNA171) for different time period (in min.) at 25 °C (lane 1-5); lane 6, 500 pmol of AC4 without any synthetic, double-stranded small RNAs (siRNA171) as control.

Fig. 3. Sequence diversity analysis of AC4 protein from various ToLCNDV. AC4 protein sequences of 23 ToLCNDV were analyzed.

(A) Analysis of AC4 protein sequence using BioEdit sequence alignment editor version 7.0. Alignment of the proteins was generated using Clustal X version 1.81.

Gonnet series was followed as protein weight matrix for amino acid alignment. The shading threshold is 90-100 % amino acid identity.

(i) AC4 protein sequences from ToLCNDVs infecting tomato and potato (Solanaceous crops) reported from India. GenBank accession numbers of the viral proteins, used for the analysis, are as follows: ToLCNDV HQ141673 in tomato (used in this study), ToLCNDV KF537780 in tomato, ToLCNDVAM850115 in potato, ToLCNDV EF068246 in tomato, ToLCNDV NC004611 in tomato, ToLCNDVU15016 in tomato, ToLCNDVAY286316 in tomato, ToLCNDV HM159454 in potato, ToLCNDVEF043231 in potato, ToLCNDVEF043230 in potato.

(ii) AC4 protein sequences from ToLCNDVs infecting Chilli pepper, eggplant (Solanaceous crops) and members of Cucurbitaceae family reported from India. GenBank accession numbers of the viral proteins, used for the analysis, are as follows: ToLCNDV EU309045 in chilli pepper, ToLCNDV EU309045 in chilli pepper, ToLCNDV in chilli pepper, ToLCNDV HQ264185 in eggplant, ToLCNDV KC545812 in cucumber, ToLCNDV AM286434 in pumpkin, ToLCNDV JN208136 in ash gourd, ToLCNDV JN129254 in pumpkin, ToLCNDV HM989845 in *Luffa acutangula*.

(iii) AC4 protein sequences from ToLCNDVs infecting Cucurbitaceae crops reported from other countries. GenBank accession numbers of the viral proteins, used for the analysis, are as follows: ToLCNDV AM747291 in bittergourd in Pakistan, ToLCNDV EF450316 in cucumber in Bangladesh, ToLCNDV GU180095 in melon in Taiwan, ToLCNDV KF749224 in zucchini in Spain, ToLCNDV KF749224 in zucchini in Spain. AC4 protein sequence from ToLCNDV (GenBank accession number HQ141673) used in this study was found to have noticeable differences at A³⁰, N⁵⁰ and M⁵⁵.

(B) Schematic presentation of development of AC4 mutants at A³⁰, N⁵⁰ and M⁵⁵ as A³⁰V, N⁵⁰H and M⁵⁵T.

(C) Structural differences in AC4, AC4 A³⁰V, AC4N⁵⁰H and AC4M⁵⁵T. (i) and (ii) Secondary structure and disorder prediction was carried out using Phyre2 server. Image colored by rainbow N → C terminus. The shaded region (ovals) showed the

changes in the AC4 secondary structure in AC4 mutants. Maximum change in secondary structure was observed in AC4N⁵⁰H.

Fig.4. Sub-cellular distribution of green fluorescent protein (GFP)-tagged ToLCNDV AC4 and its mutant proteins in *Nicotiana benthamiana* epidermal cells.

(A) AC4 and its mutants, viz., AC4-A³⁰V, AC4-N⁵⁰H and AC4-M⁵⁵T are expressed transiently by agro-infiltration, and fibrillarin tagged with monomeric red fluorescent protein (Fib-mRFP), served as a nucleolar marker. Green fluorescence derived from proteins tagged at their C-terminus with GFP: (i) AC4-GFP, (ii) AC4-A³⁰V-GFP, (iii) AC4-N⁵⁰H-GFP, (iv) AC4-M⁵⁵T-GFP. In all three cases, fluorescence was found in both the cytoplasm and around the nucleus. Red fluorescence derived from Fib-mRFP was confined to the nucleolus. Bars in the lower right corners represent 20 μ m.

(B) Characterization of the RNA silencing suppression activity of ToLCNDV AC4 and its mutant proteins AC4A³⁰V, AC4N⁵⁰H and AC4M⁵⁵T by agro-infiltration patch assay. On the left hand side of each *Nicotiana benthamiana* leaf a binary vector expressing GFP under the control of the 35S promoter was agro-infiltrated, together with an empty binary vector. On the right side of the leaf, the same binary vector expressing GFP was agro-infiltrated together with a binary vector expressing (i) tomato bushy stunt P19 protein, P19 (Positive control), (ii) AC4 protein, and its mutants (iii) AC4A³⁰V, (iv) AC4N⁵⁰H, and (v) AC4M⁵⁵T, as described (Canto et al., 2002).

(C) Western blotting based quantification of the steady-state level of GFP accumulation in the infiltrated patch using a GFP rabbit polyclonal antiserum. The lower panel represents the loading control detected by Ponceau staining.

(D) For yeast two-hybrid assays the constructs were transformed in *Saccharomyces cerevisiae* strain AH109 cells. (i) Schematic representation of interactions is shown in the plate format where pGADKT7 is denoted as AD and pGBKT7 is denoted as BD, pGBKT7-p53/pGADT7-RecT (positive control) symbolized as +, and pGBKT7-p53-/pGADT7-lamin (negative control) symbolized as -. Interaction of ToLCNDV AC4 and its mutants AC4A³⁰V, AC4N⁵⁰H, and AC4M⁵⁵T with the argonaute protein AGO4 (ii) The cells were grown on SD/-Ade/-His/-Leu/-Trp

(quadraple drop out) + X- α -gal media (AC4 and its mutants showed interaction with AGO4) streaked from (iii). (iii) Cells grown on double drop out media. (E) Western blot analysis of yeast total protein fractionated in a 10 % SDS-PAGE and probed with mouse anti-myc antiserum for expression of all the analyzed AC4 and argonaute proteins, along with the positive control, pGBKT7-p53/pGADT7-RecT, and negative control (protein isolated from non-transformed yeast cells). The lower panel represents the loading control detected by Ponceau staining.

List of Abbreviations:

ADK: Adenosine kinase

AGO: Argonaute

CMT: chromomethylase

DCL: Dicer-like

GFP: Green Fluorescent Protein

MET: methyltransferase

PTGS: Post-transcriptional gene silencing

RdDM: RNA-dependent DNA methylation

RDR: RNA-dependent RNA polymerase

RISC: RNA-induced gene silencing complexes

RITS: RNA induced transcriptional silencing

TGS: Transcriptional gene silencing

ToLCNDV: Tomato leaf curl New Delhi virus

TrAP: Transcriptional activator protein

VSR: Viral suppressor of RNA silencing

**Tomato Geminivirus encoded AC4 RNAi suppressor protein interacts with
AGO4 and preclude viral DNA methylation**

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Highlights

- Expression or silencing status of AC4 influences viral DNA cytosine methylation
- AC4 is an RNA silencing suppressor and Asn-50 in the SKNT-51 motif is crucial for suppressor activity
- Interaction with AGO4 and sub-cellular localization imply AC4 regulates PTGS and TGS
- Molecular evidence for the role of AC4 in silencing suppression

A **B** **C** **D**

Figure 1

A

B

C

Figure 2

A

ToLCNDV

(i)

(ii)

(iii)

B

C (i)

(ii)

Figure 3

A

AC4

AC4-A^{30V}AC4-N^{50H}AC4-M^{55T}

B

(i)

GFP

GFP/P19

(ii)

GFP

GFP/AC4

(iii)

GFP

GFP/AC4A^{30V}

(iv)

GFP

GFP/AC4N^{50H}

(v)

GFP

GFP/AC4M^{55T}

C

D

(i)

(ii)

(iii)

E

Figure 4