Disruption of tomato TGS machinery by ToLCNDV causes reprogramming of vascular tissue-specific TORNADO1 gene expression

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Abstract

Main conclusion Vascular development-related TRN1 transcription is suppressed by cytosine methylation in fully developed leaves of tomato. ToLCNDV infection disrupts methylation machinery and reactivates TRN1 expression - likely causing abnormal leaf growth pattern.

Abstract Leaf curl disease of tomato caused by tomato leaf curl New Delhi virus (ToLCNDV) inflicts huge economical loss. Disease symptoms resemble leaf developmental defects including abnormal vein architecture. Leaf vein patterning-related TORNADO1 gene’s (SlTRN1) transcript level is augmented in virus-infected leaves. To elucidate the molecular mechanism of the upregulation of SlTRN1 in vivo, we have deployed SlTRN1 promoter-reporter transgenic tomato plants and investigated the gene’s dynamic expression pattern in leaf growth stages and infection. Expression of the gene was delimitic in the vascular tissues and suppressed in fully developed leaves. WRKY16 transcription factor readily activated SlTRN1 promoter in varied sized leaves and upon virus infection, while silencing of WRKY16 gene resulted in dampened promoter activity. Methylation-sensitive PCR analyses confirmed the accumulation of CHH methylation at multiple locations in the SlTRN1 promoter in older leaves. However, ToLCNDV infection reverses the methylation status and restores expression level in the leaf vascular bundle. The virus dampens the level of key maintenance and de novo DNA methyltransferases SlDRM5, SlMET1, SlCMT2 with concomitant augmentation of two DNA demethylases, SlDML1 and SlDML2 levels in SlTRN1 promoter-reporter transgenics. Transient overexpression of SlDML2 mimics the virus-induced hypomethylation state of the SlTRN1 promoter in mature leaves, while silencing of SlDML2 lessens promoter activity. Furthermore, in line with the previous studies, we confirm the crucial role of viral suppressors of RNA silencing AC2 and AC4 proteins in promoting DNA demethylation and directing it to restore activated transcription of SlTRN1. Unusually elevated expression of SlTRN1 may negatively impact normal growth of leaves.

Keywords Begomovirus ORFs AC1, AC2, AC4 · DNA methylation · Disease symptom · DNA methyltransferase · DNA demethylase · Gene silencing · Leaf vein · Leaf curl · Reporter assay · ToLCNDV · Transgenic · Transcription · WRKY16

Abbreviations

CMT CHROMOMETHYLASE
DML DEMETER-LIKE PROTEINS

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Introduction

Persistent multiplication of tomato leaf curl New Delhi virus (ToLCNDV), a whitefly transmitted begomovirus, in tomato causes one of the predominant and economically important diseases known as leaf curl, resulting in constrained tomato production. The symptoms include upward or downward leaf curling, vein swelling, leaf wrinkling, and blistering with stunted and shortened internodes, somewhat mimicking leaf developmental defects.

The bipartite genome of ToLCNDV is composed of two similar-sized circular DNA, DNA-A and DNA-B. The DNA-A component contains six open-reading frames (ORFs) recognized as AC1, AC2, AC3, AC4, AV1, and AV2 (correspondingly C1, C2, C3, C4, V1, and V2 for monopartite begomovirus) encoding six proteins that help in virus replication, viral gene transcription, pathogenesis, and encapsidation, while the DNA-B has two ORFs (BC1 and BV1) required for virus movement and transmission. Genetic resistance in cultivated varieties is not common; thus, major virus management measures include whitefly control and avoidance of most susceptible cultivars. For better management, many studies are still required to understand the disease biology and the reasons behind typical symptom manifestation.

Typical symptoms of the virus infection are the outcome of interaction between multiple viral proteins and host factors which generally rule different developmental processes. For example, interaction of the pathogenicity factor, βC1, of tomato yellow leaf curl China virus with tomato ASI (ASYMMETRIC LEAVES1), a Myb transcription factor, results in altered leaf lamina symmetry, dorsiventrality, and venation patterning (Yang et al. 2008). Leaf curl viruses evolved proteins to interfere with specific developmental pathway. In N. benthamiana, the C4 gene of tomato leaf curl virus (TLCV) produced leaf curling in all infected plants, while the products of V1 and C3 triggered stunting only (Gorovits et al. 2017). We have previously reported that the expression of SITRN1 (Solanum lycopersicum TORNADO1), encoding a conserved plant-specific signaling protein, is upregulated during ToLCNDV infection in a local cultivar of tomato (Mandal et al. 2015). There are other examples of host cell signaling-related genes’ misregulation caused by the virus. These observations signify the importance of studying virus protein–host factor interaction in understanding the disease biology.

SITRN1 is a plant-specific protein of 1456 amino acids with high analogy to NOD-LRR proteins and localized in the cytoplasm. It resembles plant pathogen resistance or ‘R’ genes owing to the presence of both NBS and LRR domains, which also implies that TRN1 is involved in cell signaling. TRN1 together with the ASI plays a great role in leaf venation patterning and lateral symmetry formation. TRN1 facilitates auxin channelization for procambium cell formation leading to the differentiation of xylem and phloem cells. TRN1 mutants show altered vascular pattern formation and root development likely due to the impaired initial signaling events (Chops et al. 2006). Here, we report that SITRN1 expression level is negatively correlated to leaf maturity as well as accumulation of cytosine methylation in the promoter region. Whereas in mature leaves, SITRN1 promoter activity and expression level are increased during ToLCNDV infection. Thus, we have investigated the role of viral proteins in the reversal of methylation-mediated silencing of SITRN1 promoter in leaves of infected plants.

Epigenetic regulation of gene expression via DNA methylation in plant promoters plays a significant role in the silencing of genes whose function is not required at a specific stage of tissue development (Bartels et al. 2018). Plant DNA methylation is tissue, organelle, species, and age specific (Vanyushin 2006). DNA methylation in the CG context is common in plants, mammals, and some fungi. In higher plants, in addition to CG methylation, DNA methylation also occurs in CHG (symmetric) and CHH (asymmetric) contexts (H = A, C, or T). In Arabidopsis, DNA methyltransferase1 (MET1), an ortholog of DNMT1 in mammals, maintains CG methylations. Chromomethylase 2 and 3 (CMT2 and CMT3) and the de novo DNA methyltransferases Domains rearranged methylase 1 and 2 (DRM1 and DRM2) are mainly responsible for DNA methylations at CHG and CHH contexts. A classic example of methylation-mediated inhibition of gene expression is repression of WUSCHEL (Li et al. 2011). DNA methyltransferases CMT3 or MET1 hypermethylated WUSCHEL and inhibited its expression. Cytokinin-induced demethylation restores the gene’s expression and shoot initiation (Liu et al. 2018). Another example includes DNA hypermethylation of ERECTA family genes leading to defects in stomatal development (Wang et al. 2016). DNA methylation acts as a steady genetic mark, i.e., once the methyltransferase adds the methylation on DNA, it remains there. However, CHH methylation is termed ‘non-symmetrical’ as, during DNA replication, the opposite strand lacks a methylated cytosine and de novo methylation needs to be established after each replication cycle (Moglia et al. 2019).
DNA demethylation can act both actively by removing 5-methylcytosines by the base excision repair pathway (BER) or passively during DNA replication when a newly synthesized strand remains unmethylated or not methylated by DNA methyltransferases. The two DNA demethylases DEMETER (DME) and REPRESSOR OF SILENCING 1 (ROS1) encode DNA glycosylases and can dynamically eliminate the methylations through the BER pathway. Mutations in them can cause enhanced DNA methylations in all genomic contexts. Cytosine methyltransferases mutants of Arabidopsis show hyper susceptibility to geminivirus infections because of the limited methylation of viral DNA (Raja et al. 2008).

RNA silencing is the major antiviral mechanism found in all higher plants (Ruiz-Ferrer and Voinnet 2009; Ding 2010; Llave 2010). Several studies suggest that post-transcriptional gene silencing (PTGS) is activated against RNA viruses and transcripts produced by DNA viruses such as geminiviruses (Rodriguez-Negrete et al. 2013). Upon virus infections, double-stranded RNA molecules of viral origin are processed into small interfering RNAs (siRNAs) by the action of dicer-like proteins (DCL2, DCL4) (Elbashir et al. 2001) which mediate PTGS. Some other 24 nucleotide siRNAs, processed by DCL3, cause transcriptional gene silencing (TGS) or RNA-induced transcriptional silencing (RITS) by methylating viral DNA, thus hindering its replication. To counter the plant defense mechanism, viral proteins have evolved to interfere with the plant methylation machinery (Díaz-Pendón and Ding 2008; Raja et al. 2008). Some of the viral suppressors of RNA silencing (VSR) include viral AC2 also known as transcriptional activator protein (TrAP) (Wang et al. 2003), AC4 protein (Vanitharani et al. 2004), and V2 protein (Luna et al. 2017). The VSRs act at numerous steps of the RNA silencing pathway, thereby distressing the host defense mechanism. AC2 acts as a TGS suppressor by two mechanisms, (i) adenosine kinase inhibition (Wang et al. 2003) and (ii) reduction of proteasome-mediated degradation of S-adenosyl methionine decarboxylase 1 (Zhang et al. 2011). Some of the targets of VSRs such as AGO4 and methyltransferases like MET1, CMT3, DRM1, and DRM2 are required for methylating viral DNA. AC4 protein interacts with AGO4 and influences cytosine methylation of the viral genome (Vinutha et al. 2018). Reports suggest that AC2 protein hinders the production of S-adenosyl methionine which is a methyltransferase cofactor (Buchmann et al. 2009). Replication-associated protein (Rep) deters the expression of two key methyltransferases MET1 and CMT3 (Rodriguez-Negrete et al. 2013). Lessening of activities of methyltransferases may lead to fall of global methylation in the host genome including promoters, and misregulation of multiple host genes.

This study delved into the molecular mechanism of reversible DNA methylation on SITRN1 promoter during ToLCNDV infection. We show that AC1, AC2, and AC4 play a vital role in the suppression of the levels of maintenance and de novo DNA methyltransferases leading to transcriptional gene silencing reversal and reactivation of naturally silenced SITRN1 in mature leaves of tomato. Thus, VSRs-mediated misregulation of this crucial developmental gene could potentiate some of the typical symptoms of the disease.

Materials and methods

Plant material

Throughout the study, we have used tomato (S. lycopersicum L.) cultivar Pusa Ruby, originally released by Indian Agricultural Research Institute (IARI), New Delhi. Seeds were regularly procured from Sutton Seeds (West Bengal, India) and propagated in in-house facility. Experimental plants were grown in glass houses at 25 °C ± 2 with natural light and humidity in plastic pots containing Soilrite (Keltech Energies Ltd, Bangalore, Karnataka, India) supplemented with Suphala fertilizer (N, P, and K each at 15% by weight). For specific experiments, leaves were categorized based on their length and age as small (0.2 cm—1.0 cm, 5–10 days old), medium (1.1 cm—2.5 cm, 10–20 days old), and large (2.6 cm—4.5 cm, 20–30 days old).

Agrobacterium-mediated plant transformation

Agrobacterium-mediated transformation of tomato cotyledonary leaf explants was carried out using standard methodology. In brief, explants were collected from 7-day-old healthy seedlings grown in solid MS medium (MS salts and 3% sucrose) under aseptic condition. Agrobacterium tumefaciens LBA4404 strain harboring SITRN1 (Solyco3g112750.2) promoter fragment (− 344/+ UTR) GUS gene fusion in pCAMBIA1304 binary vector (Supplementary Fig. S1a,) was used to transform tomato explants. Agrobacterium culture was grown in LB (Luria Broth) media supplemented with 50 mg/l kanamycin and 50 mg/l rifampicin at 28 °C by continuous shaking for 48 h. Cells were harvested by centrifugation at 4 °C, resuspended in MS medium supplemented with 100 µM acetosyringone and adjusted to a final OD600 of 0.8. Cotyledonary leaves were excised of tip and petiole, and pre-incubated in MS medium supplemented with 1 mg/l zeatin for 2 days at 25 °C under 16/8 h light–dark cycles. Explants were immersed in Agrobacteria suspension for 30 min with occasional shaking and returned to the same precubination media after removal of excess liquid. Following 2 days of co-culture at 25 °C under 16/8 h light–dark cycles, the explants were transferred to regeneration media (MS medium, 1 mg/l zeatin, 250 mg/l...
ments were performed with T2-generation plants. Abaxial cotylosyringone and adjusted to a final OD600 of 0.8. Abaxial tissues obtained in T1- and T2-generation seeds and all the experiments were germinated on MS media supplemented with 25 mg/l water till the smell of bleach disappeared. Then, the seeds were sterilized with 30% commercial bleach and 0.05% Triton X 100 solution, followed by repeated washing in autoclaved sterilized water. The seeds were transferred to soilrite and kept under moist condition in culture room for hardening. Transgenic plants were planted in larger pots and maintained in glass houses for seed production.

Selection of T1 generation seeds

Seeds obtained from transgenic T0 plants were surface sterilized with 30% commercial bleach and 0.05% Triton X 100 solution, followed by repeated washing in autoclaved water till the smell of bleach disappeared. Then, the seeds were germinated on MS media supplemented with 25 mg/l hygromycin. The germination rate of 90–95% was routinely obtained in T1- and T2-generation seeds and all the experiments were performed with T2-generation plants.

Agroinfiltration of tomato leaves

Agroinfiltration technique was used to infect tomato plants with ToLCNDV and for transient gene expression and gene silencing assays. A. tumefaciens LBA4404 strain harboring clones of ToLCNDV-A (GenBank: DQ629101.1) and ToLCNDV-B (GenBank: DQ169057) genomes (LBA4404: ToLCNDV) were used for infection. Actively growing bacteria were harvested by centrifugation at 4000 g at 4 °C, following which pellet was resuspended in MES buffer (10 mM MES and 10 mM MgCl2, pH 5.6) supplemented with 100 µM acetosyringone and adjusted to a final OD600 of 0.8. Abaxial surfaces of 3 to 4 fully expanded leaves from about 20-day-old plants were infiltrated at multiple spots with approximately 100 µl of bacterial suspension in each spot, using 1 ml plastic syringe. Inoculated plants were maintained in glass house until symptoms appeared (~30 days). Similar protocol was employed to perform promoter activity assays with different constructs cloned in binary vectors pCAMBIA1304 or pPZPY112. About 40-day-old plants were used for promoter activity assays. Plants agroinfiltrated with the empty vector were used as control in all experiments.

Histochemical β-glucuronidase (GUS) staining

The GUS reporter gene activity in tissues was detected by GUS staining assays. Standard assays were performed with 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc) as the substrate. The reaction buffer consisted of 50 mM sodium phosphate buffer (pH 7.0), 1 mM EDTA, and 0.1% Triton-X100. Tissues were immersed in the buffer and incubated at 37 °C in dark for 6–10 h with intermittent shaking and monitoring. The samples were then fixed in a fixative solution consisting of 70% ethanol, 5% acetic acid, 5% formaldehyde, and 20% water for 30 min in room temperature. Subsequently, chlorophyll was removed by repeated washing with a solution consisting of glacial acetic acid, ethanol, and glycerol in 3:1:1 (by vol.) ratio and photographed using a camera (Cannon) on a compound microscope (Leica).

Measuring physiological parameters of the plant

Several physiological parameters, such as leaf stomatal conductance, atmospheric pressure, net photosynthetic rate, internal CO2, transpiration rate, vapor pressure deficit, etc., were measured using a CI-340 Handheld Photosynthesis Analyzer (CID Bio-Science, Camas, Washington, USA). Data were collected from multiple samples; average values were compared (Supplementary Table S1).

Total DNA isolation and PCR analysis

Total DNA was isolated from leaves using Plant DNAzol reagent (Invitrogen) following the manufacturer’s protocol. In brief, leaf tissues were pulverized in liquid nitrogen, and the powdered tissues were suspended in Plant DNAzol reagent (0.3 ml for 0.1 g plant tissue) supplemented with RNAse A (100 µg RNAse A/ml Plant DNAzol), mixed by gentle inversion, and incubated at 25 °C with shaking for 5 min. To the mixture 0.3 ml chloroform was added, mixed well, and centrifuged at 12,000 g for 10 min. DNA was precipitated with 100% cold ethanol from the aqueous phase and finally resuspended in TE buffer (10 mM Tris–HCl, pH 8.0, and 1 mM EDTA). Viral coat protein and movement protein-specific PCRs with CP-F/CP-R and MP-F/MP-R primer sets, respectively, and using total DNA isolated from newly emerged leaves were carried out to screen infected plants.

Total RNA isolation and RT-qPCR analysis

Total RNA was prepared from ~500 mg tissue of mock-infected (control) plants and systemic ToLCNDV-infected leaves by TRIzol reagent (Invitrogen) following the manufacturer’s protocol. To eliminate contaminating DNA the RNA samples were treated with RNase-free DNaseI (20 U/μg of DNA, Fermentas, Glen Burnie, MD, USA) for 1 h at 37 °C and RNA was further purified by phenol–chloroform extraction followed by ethanol precipitation using standard protocol. RNA integrity was checked by resolving the isolated RNA in a 1.5% agarose-TAE gel. Complementary DNA was prepared from RNA samples that had distinct 28S and 18S bands showing approximately 2:1 intensity ratio.
Five microgram total RNA was reverse transcribed to make cDNA using 200 U RevertAid reverse transcriptase (Thermo Fisher Scientific) with random hexamer (5 µM) primers in a total reaction volume of 20 µl, following the manufacturer’s protocol.

cDNAs were two-fold diluted with sterile water and 1 µl was used as template in 1 µl reaction of SYBR green-based real-time PCR using Applied Biosystems 7500 FAST machine. Three technical replicates were analyzed for each sample of cDNA. Mock infiltrated plants’ cDNA served as control. Amplification of specific products was confirmed by obtaining a single peak in melt curve analysis, first derivative of fluorescence (dF/dT) versus temperature plot. The expression level of the gene of interest, normalized to the level of EF1-α (elongation factor 1-alpha) and relative to the expression at control (described in individual experiment), was calculated for each sample using the 2−ΔΔCt method (see Supplementary Table S2).

**GUS activity assay (MUG assay)**

Leaf tissues (~ 3 sq cm) from Agroinfiltrated zones or transgenic plants were collected in a 1.5 ml centrifuge tube and grinded with liquid nitrogen. Powdered tissues were suspended in 500 µl of GUS extraction buffer (50 mM NaHPO4, pH 7.0; 10 mM 2-mercaptoethanol; 10 mM Na3EDTA; 0.1% SDS; 0.1% triton X-100) and incubated on ice for 10 min. The mixture was centrifuged at 15,000 g for 10 min at 4 °C and clarified supernatant was collected for leaf protein extracts. 50 µl of protein extract was mixed with 250 µl of GUS assay solution (GUS extraction buffer containing 2 mM 4-methyl umbelliferyl-β-D-glucuronide (4-MUG), incubated at 37 °C for 60 min, and proceeded to fluorescence measurement (excitation at 365 nm and emission at 455 nm, readings were taken for 5 s) using Varioskan flash multimode plate reader (Thermo Fisher Scientific). An aliquot of the same protein extract was diluted 50 times and concentration was determined using Bradford reagent (Himedia, Mumbai, Maharashtra, India). Data were normalized based on the protein concentration of each sample and expressed in picomoles of 4-MU (4-methylumbelliferone) released per microgram of protein per hour.

**Full-length cloning of CDS of DNA-demethylase gene**

Full-length DNA demethylase (Solyc10g083630.1.1) coding sequence was amplified from tomato cDNA pool using gene-specific primers and a high-fidelity Polymerase (KOD FX Neo, Toyobo, Kita-ku, Osaka, Japan). The 5.5 kb amplified product was gel-purified and cloned in pCAMBIA1304 binary vector utilizing the Ncol and BstEII sites downstream of the CaMV35S promoter. Positive clones were mobilized into A. tumefaciens LBA4404 for expression studies in plant.

**Methylation-sensitive PCR analysis**

Total genomic DNA was isolated from transgenic infected and uninfected tomato plants using DNAzol reagent (Thermo Fisher Scientific). Purified 20 ng DNA was digested using NlaIII restriction enzyme (New England Biolabs), that cleaves only unmethylated sites (CATG), at 37 °C for 1 h and enzyme inactivation at 65 °C for 1 h was followed. PCR primers were designed on either side of four NlaIII sites present in the promoter region of SITRN1 gene. The thoroughly digested DNA used as template to perform 30 cycles of 3 steps PCR (95 °C 30 s, 58 °C 30 s, and 72 °C 30 s) using Dream Taq DNA polymerase (NEB). Control reactions were performed with (i) undigested DNA of the same sample (no enzyme control) and (ii) amplification from a region devoid of NlaIII site (no cut site control) using both undigested and digested DNA as templates. PCR products were resolved in 2.5% agarose-TAE gel containing 1 µg/ml ethidium bromide and photographed.

**Treatment of transgenic plants with a methylation blocker**

5-Azacytidine was solubilized in 40% ethanol and 10 µM solution was freshly prepared prior to each application. One-month-old transgenic plant leaves were treated with 5-azacytidine for 20 days on both dorsal and ventral surfaces by topical application using a small cotton swab once a day. Control plants received only 40% ethanol solution. Multiple leaves of at least three biological replicates were treated for each experiment. DNA isolation and PCR reactions were carried out as mentioned above.

**CRISPRi-mediated knockdown of genes**

The CRISPRi-based pHSN6101 vector procured from Addgene (Watertown, MA, USA) which harbors SpdCas9-KRAB (Streptococcus pyogenes dCas9-Krüppel-associated box), was used for repressing SIDML2 (Solyc10g083630) and SIWRKY16 (Solyc07g056280) genes’ expressions. A 20 bp gRNA target sequence was selected within 300 bp upstream region of the transcription start site, for each of the genes and cloned into the aforementioned vector using BsoI restriction site.

Agrobacterium harboring the repression construct CRISPRi-WRKY16 (LBA4404::pHSN6101::sgRN Apsiwkry16-dCas9-KRAB) was vacuum infiltrated in transgenic leaves and also co-agroinfiltrated with ToLCNDV-A and ToLCNDV-B viral genomes to study the promoter regulation. Likewise, CRISPRi-SIDML2
(LBA4404::pHSN6I01:sgRNA_{PSIDML2-3}dCas9-KRAB) was vacuum infiltrated into mature detached PSITRN1-GUS transgenic leaves discretely and along with the Agrobacterium harboring viral proteins AC2 and AC4 expression vectors. In parallel, all the constructs were also separately infiltrated into the PSITRN1-GUS leaves to study their individual effect on promoter regulation with empty vector in Agrobacteria serving as the mock. Post 48 h of infiltration, the leaf tissues were harvested for transcript level detection and MUG assays.

**Software**

Primers were designed manually (Supplementary Table S3). OligoAnalyzer3.1 software from Integrated DNA Technologies (https://www.idtdna.com/pages/tools/oligoanalyzer) was used to analyze properties of primers, such as melting temperature, GC content, and secondary structure potential. Quantity One Basic software from Bio-Rad (www.bio-rad.com/en-uk/product/quantity-one-1-d-analysis-software) was used to quantify the intensity of PCR-band products. Upstream sequences of SITRN1 gene were downloaded from SOL Genomics Network (http://solgenomics.net/). These sequences were analyzed in MatInspector (Genomatix) to find putative cis-elements for transcription factor binding. NCBI BLAST was used for homology search and alignments. CCTop—CRISPR/Cas9 target online predictor guide (https://crispr.cos.uni-heidelberg.de/) was used to design the 20 bp gRNA target sequence used for gene silencing assay.

**Statistical analysis**

We took three different plants of same age group in all experiments. Real-time PCR analyses were performed with RNA preparations from three different plants and reactions were also done in triplicates. GUS activity assay was performed likewise with leaves of three different plants and in triplicates. Results are expressed as means ± standard deviation (SD). Two-tail Student’s t test was employed, if needed, and P ≤ 0.05 considered as significant. We have also performed two-way ANOVA test when essential and P ≤ 0.05 considered as significant.

**Results**

**SITRN1 expression is restricted to all vascular tissues of tomato**

TRN1 is linked to vein development in Arabidopsis (Cnops et al. 2006), and we have previously confirmed SITRN1 expression in leaves of tomato (Mandal et al. 2015). Therefore, we asked whether tomato TRN1 is also vascular tissue-specific. For precise monitoring of the in vivo expression pattern, we have developed TRN1 promoter-reporter transgenic lines (PSITRN1-GUS) using −344/UTR-GUS which is one of the best-studied promoter constructs (Mandal et al. 2015) (Supplementary Fig. S1a). Five primary transgenic lines derived from separate calli were obtained which are maintained for T1 and T2 generation and were utilized for subsequent experiments (Supplementary Fig. S1b). Amplification of a distinct band in hptII gene-specific PCR performed with genomic DNA isolated from leaf of these lines confirmed transgene integration (Supplementary Fig. S1c). Absence of significant amplified products in control PCRs performed with vector backbone specific primers and from wild-type plants, nullified the possibility of amplification from contaminating Agrobacterium. These plants were also expressing GUS transcripts in comparable level (Supplementary Fig. S1d). Seeds of transgenic plants were germinated on 25 mg/l hygromycin containing media prior to transfer to soil for propagation. Germination frequency of 90–95% of T0 plants was obtained, hinting insertion of multiple T-DNAs. Further experiments were performed with mostly T2-generation plants. Plants of all lines were phenotypically indistinguishable from glasshouse grown wild-type controls (Fig. 1a). Physiological parameters of transgenics were found to be equivalent to wild-type controls (Supplementary Table S1).

Histochemical staining revealed the GUS gene expression pattern, resulting from TRN1 promoter activity, in different vegetative parts (Fig. 1b, c). Stem, leaf, and root showed expression level of GUS gene in descending order (Fig. 1d). To obtain a better idea about TRN1 promoter activity in tissues, transverse sections of stem and leaf were GUS-stained. As expected, GUS activity was restricted to the vascular tissues, including vascular interfascicular and intrafascicular cambium (Fig. 1e and f) and pericycle. Staining of longitudinal section of stem confirmed heightened GUS expression within vessels (Fig. 1g). Reproductive tissues also showed high GUS expression (Supplementary Fig. S2), especially in veins. Hence, TRN1 promoter is active in vascular tissues of vegetative as well as reproductive organs of tomato.

**TRN1 gene expression pattern changes with developmental stages of leaf and virus infection**

Vascular development and reticulation are correlated to leaf growth. We have previously reported that ToLCNDV infection causes increased SITRN1 expression (Mandal et al. 2015). Thus, we intended to learn about TRN1 expression pattern in leaf growth stages during infection. Transgenic plants were infected via agroinfiltration and incubated for 1 month for disease establishment (Fig. 2a). Infection resulted in stunted plant growth, curling of leaf, and vein thickening
Fig. 1 GUS gene expression pattern in PSITRN1-GUS transgenic tomato plants. **a** Photograph of about 1-month-old control and transgenic tomato plants. **b–c** GUS-stained images of control and transgenic leaf, stem, root (b) and twig (c). **d** Relative level of GUS activity in different organs of control and transgenic plant. **e–g** Histochecmical localization of GUS expression in transverse sections of leaf (e), stem (f), and longitudinal section of stem (g) in control and transgenic plant. Control tissues did not develop color after GUS staining. MUG assay data represent average of three biological replicates. Error bars represent standard deviation (n=3). *, significant change (P ≤ 0.05) according to two-tailed Student’s t test.
Fig. 2 *SITRN1* promoter activity during ToLCNDV infection. 

**a** Photographs of about 1-month-old uninfected and virus-infected *PSiTRN1-GUS* transgenic tomato plants. 

**b** Agarose gel photograph showing resolved products of viral coat protein and movement protein gene-specific PCRs with genomic DNA of uninfected (lanes 1–3) and ToLCNDV-infected (lanes 4–6) tomato plants. 

**c** Data of MUG assay showing *SITRN1* promoter activation upon ToLCNDV infection in different lines. 

**d** GUS-stained roots of transgenic uninfected and infected plants. 

**e** GUS-stained leaves of different growth stages from control (wild-type), transgenic uninfected, and transgenic infected plants. 

**f** Quantitative assessment of *SITRN1* promoter activation in different sized leaves upon ToLCNDV infection. 

**g** Quantitative RT-PCR data showing GUS gene are highly overexpressed during ToLCNDV infection in three lines tested. 

**h** Transverse section of GUS-stained uninfected and infected transgenic leaf showing enhanced GUS staining in vascular elements in infected sample. MUG-assay data represent average of three biological replicates. Error bars represent standard deviation (s = 3). *, significant change (P ≤ 0.05) according to two-tailed Student’s t test.
with multiple open ends. Systemic infection was confirmed by virus coat protein (CP) and movement protein (MP) specific PCR analyses using total DNA isolated from leaves of uninfected and symptomatic plants (Fig. 2b). CP and MP genes were not detectable in control plants, indicating specificity of the reaction. Plants having significant infection, as indicated by prominent amplification of 750nt CP and 850nt MP amplicons, were selected for subsequent experiments. In line with our previous observation (Mandal et al. 2015) where we found augmentation of SlTRN1 gene expression in ToLCNDV infection, GUS activity was noticeably increased in leaves of all three lines tested (Fig. 2c) during the infection, with an average of 2.79 ± 0.52-fold increase \((P \leq 0.05)\) (Fig. 2c). Enhanced GUS-staining was also observed in infected roots (Fig. 2d).

To further scrutiny TRN1 expression pattern in leaves, we collected leaves of different sizes and for the simplicity of explanation grouped them into small, medium, and large leaves, and GUS expression pattern was monitored. Both GUS-staining and fluorometric MUG assays were performed to monitor GUS expression pattern in uninfected leaves as well as during infection. Interestingly, intensity of GUS-staining was most in small leaves and gradual decrease in GUS activity was noticed with progression of leaf growth. The difference in average GUS activity between small and large leaf was almost 2.5 ± 0.50 \((P \leq 0.05)\) (Fig. 2e).

ToLCNDV infection resulted in additional increment of GUS activity in small and large leaves, respectively (Fig. 2f). GUS activity in small, medium, and large leaves was increased in the order of 2.6 ± 0.50 \((P \leq 0.05)\), 3.06 ± 0.29 \((P \leq 0.05)\), and 3.49 ± 0.45 \((P \leq 0.05)\) folds, respectively, during the infection. Tissue staining also indicated heightened GUS activity in vascular bundles of mature leaves, further indicating TRN1 promoter activation in large leaves during infection (Fig. 2h). These results hint that SlTRN1 gene expression is tightly regulated during leaf growth, while virus disrupts the regulation pattern.

**WRKY transcription factor mediates augmentation of transcription from SlTRN1 promoter and GUS transgene expression**

To investigate the mechanism of heightened GUS expression, we first analyzed the GUS transcript level, which should provide information about the SlTRN1 promoter activity. RT-dependent real-time PCR was carried out with GUS gene-specific primers. The analysis confirmed upregulation of GUS transcript level in all three lines tested (2 folds, \(P \leq 0.05\), average of three lines) upon ToLCNDV infection (Fig. 2g). Thus, it is apparent that heightened GUS expression in PSiTRN1-GUS plants is the result of enhanced transcript level generated from activated PSiTRN1 transcription. The promoter harbors two prominent W-box elements (Supplementary Fig. S1a). We had previously shown (Mandal et al. 2015) that WRKY16 expression was increased during the infection and WRKY16 readily activated SlTRN1 promoter. Here, we show that Agroinfiltration of WRKY16 into PSiTRN1-GUS transgenic plants also activates the promoter, resulting 2.1 ± 0.18-fold \((P \leq 0.05)\) upregulation of GUS transplant level (Fig. 3a), and 2.13 ± 0.45-fold \((P \leq 0.05)\) and 2.29 ± 0.16 fold \((P \leq 0.05)\) increase in GUS activities in small and large leaves, respectively (Fig. 3b).

Infiltration of a set of NAC transcription factors (Bhattacharjee et al. 2017) or treatment with abiotic stress had no effect (Supplementary Fig. S3b). We have also checked the regulation of SlTRN1 promoter under the influence of WRKY16 translocation factor during virus infection and found 1.55 ± 0.09-fold \((P \leq 0.05)\) upregulation of GUS activity in infection (Fig. 3c). Agroinfiltration of WRKY16 into infected leaves resulted in additional increment of GUS activity (from average 1376.11 pmol MU to 2144.09 pmol MU produced per µg protein/h; blue bars, Fig. 3c), which is also evident from comparison of color intensity of GUS-stained uninfected and infected transgenic leaves (Fig. 3d).

This observation is further corroborated by CRISPRi-mediated knockdown of WRKY16 and simultaneous expression of viral genome. Data presented in Fig. 3e indicate effective downregulation of SIWRKY16 gene expression \(0.53 \pm 0.18\)-fold, \(P \leq 0.05\) that occurred upon infiltration of Agrobacterium harboring the repression construct WRKY16-CRISPRi. Downregulation of SIWRKY16 resulted in small but significant loss in SlTRN1 promoter activity \(0.6 \pm 0.14\)-fold, \(P \leq 0.05\). However, simultaneous expression of the viral genome enhanced the promoter activity (Fig. 3f). Hence, WRKY16-mediated transcriptional regulation can act synergistically to the infection, or an alternative mechanism of promoter regulation is activated during ToLCNDV replication.

**Methylation pattern of SlTRN1 promoter is regulated during growth and ToLCNDV infection**

Differential DNA methylation of the genome is an integral component of the regulation of gene expression in angiosperms. The exclusive expression pattern of SlTRN1 gene in leaf developmental stages and also during ToLCNDV infection in transgenic plants prompted us to investigate whether dynamics of promoter methylation plays an important role in regulating the gene expression under different conditions. We opted for the methylation-sensitive PCR analysis (Dasgupta and Chaudhuri 2019) to identify the cytosine methylations in the promoter region...
of the SITRN1 gene. It harbors four CATG sequences, recognition site of NlaIII restriction enzyme, within 500nt upstream to the translation start site (Fig. 4a, b). NlaIII cleaves only unmethylated CATG sites (Guzmán-Benito et al. 2019). We have utilized this enzyme in methylation-sensitive PCR analysis of genomic DNA isolated from different sizes of leaves of wild-type plants, and uninfiltrated as well as ToLCNDV-infected PSITRN1-GUS transgenic tomato plants. In wild-type and uninfiltrated samples, the band intensities of PCR products obtained from NlaIII cut genomic DNA were varied in the order of large > medium > small leaves (Supplementary Fig. S4a, Fig. 4c and 4e). Hinting, methylations in all four sites were more common in larger leaves compared to the small and medium leaves. Intriguingly, meager amount of amplification was detected in NlaIII digested infected samples (Fig. 4d), and greater inhibition of amplification was observed in samples collected from large leaves (Supplementary Fig. S4a, Fig. 4c and 4e). Thus, infection caused inhibition or removal of methylations in all four sites tested, in all sized leaves. Equivalent intensities of bands in no enzyme controls affirmed that equal amount of DNA was used in each sample in this experiment. To confirm the integrity and quality of the DNA used in these experiments, we performed the ‘no cut site’ control reactions with all enzyme-treated and no-enzyme-treated templates. The equal intensity of PCR products in each sample validates that high quality of the DNA was always used. A control experiment is performed with CATG methylation specific enzyme, CviAII which cleaves both unmethylated and methylated CATG unlike NlaIII. Observation of faint or negligible bands in enzyme-treated samples of varied sized leaves (small, medium, large) confirmed that CviAII cleaves both methylated and unmethylated CATG, so augmented cytokine methylation in large leaves does not affect the band intensities (Supplementary Fig. S4b). Collectively, these data show that SITRN1 promoter accumulates more methylation with aging of a leaf, while infection obliterates methylation marks at all tested sites, irrespective of the age of the leaf.

**Treatment with a methylation blocker abolishes methylation marks at NlaIII sites**

To examine the effect of a methylation blocker on the promoter methylation we have treated plants with 5-azacytidine, which inhibits methyltransferase activity leading to hypomethylation of genomic DNA. Leaves of varied age were regularly smeared with either 5-azacytidine or the solvent only as untreated control. NlaIII digested and no enzyme control genomic DNA from untreated and treated plants were subjected to methylation-sensitive PCR analysis (Fig. 5a). In plants treated with methylation blocker, methylation marks were not detectable in any of the 4 NlaIII sites, as observed by the absence of bands in treated and enzyme digested samples, while in untreated samples, the band intensity was increased from small to large leaves (Fig. 5a, right panel vs. left panel; Fig. 5b). Other control reactions were similar to that have been described in the previous section. Next, we investigated the effect of 5-azacytidine treatment on promoter activation. Significant higher reporter activity in the order 2.87 ± 0.13 \((P \leq 0.05)\), 3.12 ± 0.26 \((P \leq 0.05)\), and 4.14 ± 0.28 \((P \leq 0.05)\) folds in small, medium, and large leaves, respectively, was noted (Fig. 5c), while maximum activation occurred in larger leaves. Intense blue precipitation in 5-azacytidine-treated large leaves compared to mock treated ones confirmed the recrudescence of promoter (Fig. 5d). These results further validate that accelerated methylation at CATG sites in SITRN1 promoter leads to suppression of promoter activity in mature leaves.

**DNA methyltransferase and demethylase expression pattern correlates with SITRN1 promoter methylation level in varied sized leaves and ToLCNDV infection**

The differential level of methylations found in the promoter region of varied sized leaves as well as in virus-infected plants hinted that altered regulation of DNA methyltransferases and/or DNA demethylase was responsible for such variation in methylation intensity. Hence, we set out to investigate some of these genes’ expression levels during leaf growth. Interrogation of the tomato genome yielded four DNA methyltransferases (SICMT4, Solyc08g005400; SIMET1, Solyc11g030600; SICMT2, Solyc12g100330 and SIDRM5, Solyc02g062740) which are homologous to Arabidopsis CHROMOMETHYLASE3 (CMT3), and DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2). Three DNA demethylases DEMETER-LIKE proteins (SIDML3, Solyc11g007580; SIDML1, Solyc09g009080 and SIDML2, Solyc10g083630) which are homologous to...
Arabidopsis DEMETER (DME) and REPRESSOR OF SILENCING1 (ROS1) were also identified (Supplementary Table S2). Interestingly, these demethylases are differentially expressed in geminivirus infection (Rodríguez-Negrete et al. 2013). RT-dependent quantitative PCR analysis was performed to detect the expression level of these genes in three different sized leaves of transgenic plants (Fig. 6a). Expression levels of 3 methyltransferases, SiDRM5, SiMET1, and SiCMT2, were significantly higher in large leaves, $4.20 \pm 0.56 (P \leq 0.05)$, $4.80 \pm 0.65 (P \leq 0.05)$, and $2.66 \pm 0.85 (P \leq 0.05)$ folds, respectively, compared to small leaves. Concomitant decrease in expression level of three DNA demethylases (SiDML3, SiDML1, and SiDML2), in the order of $0.18 \pm 0.02 (P \leq 0.05)$, $0.14 \pm 0.07 (P \leq 0.05)$,
Fig. 5 Promoter methylation and activity analyses after treatment with a methylation blocker. 

- **a** Agarose gel photograph showing profile of methylation-sensitive PCR products obtained with genomic DNA isolated from untreated and 5-azacytidine-treated transgenic plant leaves. Amplification profile from a genomic region with no NlaIII sites used as loading control.

- **b** Bar graph showing quantification (n=3) of methylation-sensitive PCR products in 5-azacytidine-treated leaves. 

- **c** GUS activity assay showing magnitude of SlTRN1 promoter reactivation upon treatment with 5-azacytidine in different sized leaves. 

- **d** GUS staining of 5-azacytidine untreated and treated mature leaves. Intense staining upon treatment also confirms SlTRN1 promoter reactivation. MUG-assay data represent average of three biological replicates. Error bars represent standard deviation (n=3). * indicates significant change (P≤0.05) according to two-tailed Student’s t test, and ANOVA analysis (b).
and 0.078 ± 0.04 (P ≤ 0.05) folds, respectively, in large leaf compared to the small leaf, was also noted (Supplementary Table S2). Thus, we concluded that differential expression of methyltransferases and demethylases likely rule the methylation status at CATG sites in different sized leaves.

We have also performed RT-dependent quantitative PCR analysis to detect the expression level of these genes in mature leaves of transgenic control and virus-infected plants. As expected, levels of three DNA methyltransferases, \textit{SIDRM5}, \textit{SIMET1}, and \textit{SIDMT2}, were significantly decreased by 0.24 ± 0.06 (P ≤ 0.05), 0.46 ± 0.12 (P ≤ 0.05), and 0.35 ± 0.09 (P ≤ 0.05) folds, respectively, in infection (Fig. 6b). Concurrently, transcript levels of two DNA demethylases, \textit{SIDML1} and \textit{SIDML2}, were augmented by 2.03 ± 0.84 (P ≤ 0.05) and 3.15 ± 0.79 (P ≤ 0.05) folds, respectively, in tested samples (Fig. 6b, Supplementary

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**Fig. 6** Expression level of four DNA methyltransferase and three DNA demethylases under normal physiological condition and various treatments. \textbf{a-c} Relative expression level in PSiTRN1-GUS plants of four DNA methyltransferases and three DNA demethylases tested in varied sized leaves (a), in uninfected and ToLCNDV-infected plants (b), and upon transient overexpression of ToLCNDV genes (c). Pooled medium and large leaves were used in experiments presented in \textbf{b} and \textbf{c}. AC1, replication initiator (Rep); AC2, transcriptional activator (TrAP); AC3, replication enhancer (REn), and AC4, pathogenicity determinant proteins were used. RT-PCR data represent average of three biological replicates. Error bars represent standard deviation (n=3), *, significant change (P≤0.05) according to two-tailed Student’s t test.
Table S2). These observations suggest that ToLCNDV infection causes both decreased methylation and enhanced demethylation for maintaining *SITRN1* promoter as hypomethylated and fully active in all leaves.

**Viral replication initiator protein AC1, transcriptional activator protein AC2, and pathogenicity determinant protein AC4 are the major regulators of DNA methyltransferase and demethylase.**

We have also designed experiments for identifying the viral factors causing misregulation of methyltransferases and demethylases. Full-length ORFs of replication initiator protein (Rep)-AC1, transcriptional activator protein (TrAP)-AC2, replication enhancer protein (Ren)-AC3, and pathogenicity determinant protein-AC4 were agroinfiltrated in tomato leaves. Relative expression levels of methylases and demethylases were determined by RT-dependent qPCR analysis on isolated RNA from infiltrated leaves. We observed *SIDRM5, SIMET1* and *SICMT2* downregulation by 0.86 ± 0.03-fold, *P* ≤ 0.05; 0.47 ± 0.04-fold, *P* ≤ 0.05; and 0.48 ± 0.15-fold, *P* ≤ 0.05, respectively, in AC1; and 0.75 ± 0.12-fold, *P* ≤ 0.05; 0.86 ± 0.01-fold, *P* ≤ 0.05; and 0.63 ± 0.05-fold, *P* ≤ 0.05, respectively, in AC4 infiltrated leaves. On the other hand, we had noted *SIDML2* upregulation by 3.44 ± 0.90-fold, *P* ≤ 0.05 and 3.28 ± 0.21-fold, *P* ≤ 0.05, respectively, in AC1 and AC4 infiltration. Additionally, *SIDML1* was significantly upregulated (2.28 ± 0.06-fold, *P* ≤ 0.05) in AC4 infiltrated leaf (Fig. 6c, and Supplementary Table S2). While AC2 infiltrated samples show downregulation of four methyltransferases *SICMT4, SIDRM5, SIMET1*, and *SICMT2* (0.85 ± 0.02-fold, *P* ≤ 0.05; 0.51 ± 0.04-fold, *P* ≤ 0.05; 0.8 ± 0.02-fold, *P* ≤ 0.05; and 0.4 ± 0.02-fold, *P* ≤ 0.05, respectively) and upregulation of three demethylases *SILDL3, SIDML1*, and *SIDML2* (1.24 ± 0.04-fold, *P* ≤ 0.05; 1.73 ± 0.05-fold, *P* ≤ 0.05; and 1.81 ± 0.06-fold, *P* ≤ 0.05, respectively). Not much change in gene expression pattern was seen upon AC3 infiltration. These observations are in line with our previous findings about expression pattern of methyltransferases and demethylases during infection. However, a closer inspection of the overall data suggests that only *SIDML2* was upregulated in three, AC1, AC2, and AC4, infiltrations, and the magnitude of the upregulation of *SIDML2* was more compared to other demethylases we have tested (Supplementary Table S2).

**Overexpression of DNA demethylase (*SIDML2*) restores hypomethylation of *SITRN1* promoter and expression**

We have investigated the effect of transient overexpression of a demethylase on *TRN1* promoter activation and GUS expression in large leaves. We have selected the DNA demethylase *SIDML2* for this purpose, since its expression was abruptly changed in large leaves, during infection and AC1, AC2 and AC4 infiltrations. Full-length *SIDML2* (Fl *SIDML2*) was cloned (Fig. 7a, inset) and Agroinfiltrated in multiple spots in large leaves for transient overexpression. Two days post-infiltration, *SIDML2* level was upregulated by 8.64 ± 0.12-fold (*P* ≤ 0.05) (Fig. 7a). Subsequently, DNA was isolated from the infiltrated tissue and subjected to methylation-sensitive PCR analysis. Comparison of normalized PCR-band intensities in only vector and Fl *SIDML2* infiltrated *Nla*III digested samples indicated Fl *SIDML2* infiltration nearly obliterated methylations at CATG sites (Fig. 7b, c). Darker bands obtained with only vector infiltrated samples are also indicative of usual methylations at tested CATG sites (Fig. 7b, c). All controls were similar to the reactions described in previous sections.

Next, we investigated the effect of *SIDML2* expression on *SITRN1* promoter activation by monitoring the GUS activity in infiltrated leaves. Significant higher reporter activity in all categories of leaves was apparent (Fig. 7d), while maximum activation occurred in larger leaves. The magnitude of increase was in the order of 2.68 ± 0.13 (*P* ≤ 0.05), 2.93 ± 0.26 (*P* ≤ 0.05), and 3.75 ± 0.28 (*P* ≤ 0.05) folds in small, medium, and large leaves, respectively. Intense blue coloration of *SIDML2*-infiltrated leaves compared to only vector (vector control) infiltrated samples confirmed reactivation of the promoter (Fig. 7e). These data strongly support that demethylation of *SITRN1* promoter via activation of demethylase could activate the promoter as well as restoration of promoter activity in older mature leaves as observed during infection.

**Viral AC2 and AC4 disrupt the epigenetic regulation and expression of *SITRN1* promoter**

We have already shown that viral AC2 and AC4 proteins could majorly control the level of DNA methyltransferases’ and demethylases’ expression in leaves. Next, we investigated the effect of transient overexpression of AC2 and AC4 in hypomethylation and restoration of *SITRN1* promoter activity. Upon Agroinfiltration into *PSITRN1*-GUS plants, high levels of AC2 and AC4 expression, 6.15 ± 0.12 (*P* ≤ 0.05) and 6.17 ± 0.11 (*P* ≤ 0.05) folds, respectively, were noticed (Fig. 8a). Methylation-sensitive PCR analysis was performed with the isolated genomic DNA from these leaves. Faint amplification bands obtained with the DNA isolated from infiltrated and *Nla*III digested samples were indicative of almost null methylations in the *TRN1* promoter. While higher band intensity in blank vector infiltrated leaves confirmed existence of usual methylations at CATG sites in the promoter (Fig. 8b, c). All other control reactions were identical to the reactions described in preceding sections.
Fig. 7 Restoration of promoter activity by DNA demethylase in mature leaves. **a** qRT PCR data showing relative level of *SidML2* transcript expression in leaves of PSITRN1-GUS transgenic tomato plants agroinfiltrated with pCAMBIA1304 (vector control) or pCAMBIA1304:SidML2 (Fl SidML2). Fl SidML2-infiltrated leaves have higher level of *SidML2* expression. Inset, agarose gel photograph showing full-length *SidML2*, 6 kb, digested product obtained from a pCAMBIA1304:SidML2 clone. **b** Agarose gel photograph showing resolved bands of methylation-sensitive PCR products obtained with genomic DNA isolated from vector control and Fl SidML2-infiltrated transgenic tomato leaves. Result of quantification and comparison of band intensities is shown in (e). Amplification of a genomic region with no NlaIII sites used as loading control. **d** MUG-assay data showing magnitude of SITRN1 promoter activation upon Fl SidML2 agroinfiltration in different sized leaves. **e** Vector control and Fl SidML2 agroinfiltrated GUS-stained transgenic mature leaves shown in duplicate. Intense staining upon *SidML2* expression confirmed reactivation of SITRN1 promoter. MUG assay and RT-PCR data represent average of three biological replicates. Error bars represent standard deviation (n = 3). *, significant change (P ≤ 0.05) according to two-tailed Student’s t test, and ANOVA analysis (c).
We also studied the effect of AC2 and AC4 expression on SlTRN1 promoter activation. MUG assays were performed after infiltration of AC2 and AC4 in small and large leaves. Results indicated enhanced promoter activation in these leaves (Fig. 8d). The intensity of increase in AC2 and AC4 infiltrated samples was in the order of 1.84 ± 0.10 (P ≤ 0.05) and 1.92 ± 0.19 (P ≤ 0.05) folds, respectively, in small leaves, and 3.90 ± 0.07 (P ≤ 0.05) and 4.12 ± 0.09 (P ≤ 0.05) folds, respectively, in large leaves. Thus, the degree of upregulation was more in the large leaves compared to the small ones. Intense blue coloration of infiltrated leaves compared to only vector infiltrated (vector control) samples also proved promoter activity was restored (Fig. 8e). These data clearly demonstrate that AC2 and AC4 proteins actively alter the natural methylation pattern of the SlTRN1 promoter, which leads to reactivation of SlTRN1 promoter particularly in older mature leaves during infection.

Since SIDML2 had profound effect in reactivation of silent SlTRN1 promoter, we investigated whether AC2 and AC4's effects are also mediated only via SIDML2. Expression of SIDML2 in PSITRN1-GUS plants was repressed utilizing CRISPRi-SIDML2 system. Typically, 0.48 ± 0.18-fold (P ≤ 0.05) repression in mRNA expression was noticed (Fig. 8f). SlTRN1 promoter activity was reduced to the extent of 0.49 ± 0.06-fold (P ≤ 0.05) in mature leaves upon downregulation of SIDML2. Although AC2 or AC4's effect seems to be additive to that of SIDML2, their expression in SIDML2-knockdown leaves could still alleviate silencing of the SlTRN1 promoter (Fig. 8g)—signifying, the effect of the viral proteins on cellular methylation machinery is complex and inherently multivariate.

Altogether, we have presented in vivo data reaffirming our previous observation of augmentation of vascular tissue-specific expression of TORNADO1 gene in tomato during ToLCNDV infection. Interestingly, the expression of SlTRN1 remains unabated in mature leaf during infection, which otherwise becomes transcriptionally silenced. Methylation at specific sequences of the promoter was found to be the primary determinant for suppressing the gene expression in fully developed leaf; however, ToLCNDV infection interferes with the plant DNA methylation maintenance machinery to subdue the transcriptional gene silencing for reactivation of the gene expression.

**Discussion**

Previously, mostly via transient expression analyses, we had analyzed the mechanism of upregulation of the SlTRN1 gene in tomato plant during ToLCNDV infection (Mandal et al. 2015). Here, we have delved into the mechanism of its regulation in vivo during ToLCNDV infection. One of the efficient methods of studying a gene’s regulation without affecting the normal function of the gene is to utilize a promoter-reporter transgenic. Thus, we had proceeded to generate PSITRN1-GUS transgenic tomato plants. Results presented here represent cumulative data obtained from the analysis of multiple transgenic lines.

**Arabidopsis trn1** mutants had defective lamina symmetry, growth, and anomalous venation patterning (Cnops et al. 2006). Histochemical staining of transverse sections of vegetative and reproductive organs of SlTRN1 promoter transgenic plants also confirmed the limitation of TRN1 expression in vascular tissues (Figs. 1e-g and Supplementary Fig. S2), indicating that spatial expression pattern of TRN1 is conserved in different species. Another interesting observation was the temporal regulation of the SlTRN1 gene expression in leaves of different growth stages. We found weakened promoter activity with the progression of leaf growth (Fig. 2e-f). This observation indicated that SlTRN1 expression is essential in the growing leaves or the younger parts and dispensable in the fully expanded and developed regions. The phenomenon is not related to transgene suppression since similar pattern of endogenous SlTRN1 expression has also been noticed (Supplementary Fig. S3a). Also, multiple lines exhibited the same pattern of SlTRN1 regulation, which proves suppression of expression is independent of the integration context of the transgene.

In line with the previous study from this laboratory (Mandal et al. 2015), higher SlTRN1 gene expression in ToLCNDV-infected transgenic plant leaves has been confirmed by quantitative real-time PCR analysis, fluorometric MUG assay, and histochemical GUS staining experiments (Fig. 2c-h). Restoration of SlTRN1 expression in mature leaves upon infection (Fig. 2e) was the most significant observation, which is supported by the fact that the degree of augmentation of promoter activity upon virus infection was higher in large leaves compared to that in the small leaves (Fig. 2f). Thus, we can safely infer that the virus infection can reverse the effect of silencing of SlTRN1 gene expression in mature leaves. WRKY16 transcription factor could readily induce expression of PSITRN1-GUS (Fig. 3a), similar to our previous transient assays. Lower PSITRN1-GUS activity in WRKY16-repressed leaves further validated the vital role of WRKY16 in maintaining the SlTRN1 promoter activity (Fig. 3f).

The distinctive expression pattern of the SlTRN1 gene in transgenic plants in developmental stages and during ToLCNDV infection tempted us to ponder on whether promoter methylation played an important role in regulating the gene expression. Methylation-sensitive PCR analyses indeed showed that (i) SlTRN1 proximal promoter region is subjected to CATG (CHH) methylations at multiple sites, (ii) augmented cytosine methylation is present in large leaves as compared to small ones, (iii) promoter activity is negatively correlated to leaf growth and methylation intensity,
and (iv) almost absence of methylation marks in all sites tested as well as restoration of promoter activity in fully grown leaves are correlated to ToLCNDV infection (Fig. 4c, 4d and 2e). We have used a methylation blocker and proved that the variations in the amplicon band intensities are the results of changed methylations and showed restoration of promoter activity. Thus, *SITRN1* promoter is repressed by CATG methylations (Fig. 4). Increased methylation is not due to tissue culture or the context of integration of the transgene, because wild-type plants and different transgenic lines exhibited similar pattern (Supplementary Fig. S4a). In fact, A1 and A2 amplicons, harboring two and one CATG sites, respectively, should be amplified only from the endogenous mature leaves, shown in duplicate, agroinfiltrated with vector control, AC2 and AC4 constructs. Intense staining in AC2 and AC4 agroinfiltrated leaves confirms promoter reactivation. f qRT PCR data indicating downregulation of *SIDML2* gene expression upon infiltration of *Agrobacterium* harboring repression construct CRISPRi-SIDML2. g MUG-assay data showing downregulation of *SITRN1* promoter activity upon infiltration of CRISPRi-SIDML2. Promoter activity in SIDML2-repressed leaves increases upon expression of AC2 or AC4 protein. AC2 and AC4 further upregulate *SITRN1* promoter activity in conjunction with FlSIDML2 in transgenics in comparison to solitary FlSIDML2 infiltration. MUG assay and RT-PCR data represent average of three biological replicates. Error bars represent standard deviation (*n* = 3). *, significant change (*P* ≤ 0.05) according to two-tailed Student’s *t* test and ANOVA analysis (e) and Table S2). Similar upregulation in the level of four methyltransferases, *SmelMET1*, *SmelCMT2*, *SmelCMT3a*, and *SmelDRM3*, in *Solanum melongena* during fruit development was observed (Moglia et al. 2019). Therefore, the expression pattern of methylation and demethylation-related genes observed in our study agrees with the previous observations, and differential promoter methylation could be the major player for the regulation of *SITRN1* expression in small and large leaves.

We have shown previously that WRKY16 interaction with *SITRN1* promoter region results in activated transcription. Two W-boxes are present midway between two *NlaIII* sites (Supplementary Fig. S1a and S5), so may be CATG methylation (*NlaIII* site) is not hindering WRKY binding, but overall promoter DNA methylation may preclude WRKY from binding in the promoter of the *SITRN1* gene. *Arabidopsis* plants having mutations in cytosine methylation machinery showed differential expression of pathogenesis-related genes. CHH methylation pattern is also dependent on pathogen types. An earlier report showed Viroids induce hypomethylation of the promoter region of rRNA genes resulting in increased expression (Martinez et al. 2014). Seemingly, a direct link exists between pathogenesis and the level of DNA methylation. Since *SITRN1* promoter activity is restored in fully grown leaves after ToLCNDV infection, we questioned whether the virus is meddling with the methyl cycle during infection by lessening or enhancement of some of the key regulatory enzymes that help to maintain methylation in mature leaves. Infection with ToLCNDV in the transgenic plants reduced the expression level of key maintenance and de novo DNA methyltransferases with concurrent upregulation in transcript levels of two DNA demethylases in tested samples (Fig. 6b, Supplementary Table S2), essentially reversing methyl cycle gene expression levels in mature leaves. However, unlike the observations with mutant *MET1* plants (Yang et al. 2019), we have detected hypomethylation of CHH sites in virus-infected leaves when *MET1* was downregulated. Thus, the major reason for the hypomethylation phenomenon observed here may be attributed to the upregulation of demethylases.

Activation of the host DNA methylation pathway to methylate the virus genome to suppress its expression is a crucial defense strategy, while a virus recruits its proteins to counter the methylation pathways. Incidentally, the methylation status of several host genes is also compromised during an active infection. Proteomics studies of geminivirus-infected plants followed by VIGS-mediated specific gene silencing and infection analyses confirmed the role of the RNA-directed DNA methylation (RdDM) pathway in host defense against the virus (Zhang et al. 2011; Zhong et al. 2017). On the other hand, several reports confirmed viral proteins as suppressors (VSRs) of DNA methylation or RdDM. Examples include C4 or AC4 (Ismayil et al. 2018; Vinutha et al. 2018) and *Candida* et al. 2014).
to the region proximal to the transcription start sites, the noticed the phenomenon in promoter transgene in multiple or transposable sequence silencing, because we have also methylated in fruits and chilling methylome. Some sRNAs sequences. These repeat regions are found to be highly Miniature inverted-repeat transposable elements (MITE) up or downstream of the gene is rich in repeats, including AC2 and AC4 caused hypomethylation and restoration of activity of the SITRN1 promoter in fully grown leaves (Fig. 8d and e).

Collectively, among the methyl cycle enzymes, SIDML2 seemed to be the major mediator of infection-dependent reversible methylation of SITRN1 promoter due to its abrupt changes in expressions in mature leaves, during ToLCNDV infection and AC1, AC2 as well as AC4 infiltrations (Supplementary Table S2). SIDML2 knockout plants show absence of DNA demethylation, thus inhibiting the expression of several tomato fruit ripening genes due to global hypermethylation (Lang et al. 2017; Huang et al. 2019). Consequently, by transient infiltration assays, we found that SIDML2 overexpression creates hypomethylation of the SITRN1 promoter in fully grown leaves and refurbishes promoter activity in all leaves, while SIDML2 suppression reduced SITRN1 promoter activity probably due to hypermethylation (Fig. 8g). Altogether these data conclusively prove that demethylation of promoter via activation of a demethylase during ToLCNDV infection reactivates, otherwise transcriptionally suppressed, SITRN1 gene and restores expression in fully grown leaves. However, coinfiltration of AC2 and AC4 in SIDML2 deprived plants portrayed a more complex scenario—multiple methylases and demethylases might control SITRN1 promoter demethylation during infection. A schematic representation of the sequence of events occurring in the PSITRN1-GUS transgenic tomato plant in control and ToLCNDV infection condition is shown in Supplementary Fig. S5.

A search in the tomato epigenome database (http://ted.bti.cornell.edu/cgi-bin/epigenome/home.cgi) of methylation pattern of SITRN1 genomic region revealed that 5 kb up or downstream of the gene is rich in repeats, including Miniature inverted-repeat transposable elements (MITE) sequences. These repeat regions are found to be highly methylated in fruits and chilling methylome. Some sRNAs also seemed to be aligned to the repeat sequences. However, silencing of SITRN1 may not be linked to the adjacent repeat or transposable sequence silencing, because we have also noticed the phenomenon in promoter transgene in multiple transgenic lines. Although small RNAs were not matched to the region proximal to the transcription start sites, the proximal promoter region in the leaf undergoes localized cytosine methylation at CG, CHH, and CHG sites at specific regions. This is also raising the possibility that reversible methylations occur at additional sites which have not been analyzed yet.

The TGS and RNA-directed DNA methylation (RdDM) pathways have long been implicated in leaf curl disease severity. Transgenic expression of RNA-dependent RNA polymerase1 in N. benthamiana caused reduced susceptibility toward Tomato leaf curl virus (ToLCNDV) infection (Prakash et al. 2020). C4-mediated suppression of SAMS activity facilitated viral multiplication and determined symptom intensity (Ismayil et al. 2018). The C4 protein of tomato leaf curl Yunnan virus interacts with DRM2 to suppress methylation of the viral genome and a virus with a mutation in the C4 DRM2 interaction region produced only milder symptoms (Mei et al. 2020). C4 also indirectly inflicts accumulation of CyclinD1.1 in N. benthamiana plant causing abnormal cell division (Mei et al. 2018). AC4 protein disrupts the auxin biosynthesis/signaling pathway, visa-a-vis viral infection upregulates miR167 and miR393, further affecting auxin signaling in leaf (Vinutha et al. 2020). Besides, exogenous auxin application somewhat deters disease symptoms, signifying suppression of auxin signaling is linked to typical symptom manifestation (Vinutha et al. 2020). Auxin canalization is one of the important factors in vein development and TRN1 protein involved in sensing canalization signal and vein reticulation. Enhanced or ectopic expression of auxin signaling genes leads to leaf developmental defect. The phenotype of PINOID, enhancer of polar auxin transport, overexpressing plants resembled that of auxin transport or sensitivity mutants (Saini et al. 2017) including stunted rosette formation. Overexpression of SITIR1 results in abnormal leaf development having a smaller leaf length-to-width ratio in tomato (Ren et al. 2011). Thus, it is likely that misregulation of SITRN1 during ToLCNDV infection can inflict leaf developmental defects (Supplementary Fig. S5) and contributes to the typical leaf curl symptom manifestation.

**Author contribution statement**

SC and PK designed all experiments. SC performed majority of the experiments. AM performed CRISPRi-related studies. SB, RD, and A Mandal provided some reagents and assisted in conducting the experiments. SC and PK wrote the paper. PK supervised the study. All authors have seen the manuscript, provided input, and confirmed submission.

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Data availability Additional data supporting the findings in this study are available in the Supplementary Information of this article. The raw datasets are available from the first author or corresponding author on reasonable request.

Declarations

Conflict of interest The authors have no financial or non-financial interests to declare that are relevant to the content of this article.

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