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ORIGINAL ARTICLE

Tomato auxin biosynthesis/signaling is reprogrammed by the geminivirus to enhance its pathogenicity

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Abstract

Main conclusion **Tomato leaf curl New Delhi virus-derived AC4 protein interacts with host proteins involved in auxin biosynthesis and reprograms auxin biosynthesis/signaling to help in viral replication and manifestation of the disease-associated symptoms.**

Abstract Perturbations of phytohormone-mediated gene regulatory network cause growth and developmental defects. Furthermore, plant viral infections cause characteristic disease symptoms similar to hormone-deficient mutants. Tomato leaf curl New Delhi Virus (ToLCNDV)-encoded AC4 is a small protein that attenuates the host transcriptional gene silencing, and aggravated disease severity in tomato is correlated with transcript abundance of AC4. Hence, investigating the role of AC4 in pathogenesis divulged that ToLCNDV-AC4 interacted with host TAR1 (tryptophan amino transferase 1)-like protein, CYP450 monooxygenase—the key enzyme of indole acetic acid (IAA) biosynthesis pathway—and with a protein encoded by senescence-associated gene involved in jasmonic acid pathway. Also, ToLCNDV infection resulted in the upregulation of host miRNAs, viz., miR164, miR167, miR393 and miR319 involved in auxin signaling and leaf morphogenesis concomitant with the decline in endogenous IAA levels. Ectopic overexpression of ToLCNDV-derived AC4 in tomato recapitulated the transcriptomic and disruption of auxin biosynthesis/signaling features of the infected leaves. Furthermore, exogenous foliar application of IAA caused remission of the characteristic disease-related symptoms in tomato. The roles of ToLCNDV-AC4 in reprogramming auxin biosynthesis, signaling and cross-talk with JA pathway to help viral replication and manifest the disease-associated symptoms during ToLCNDV infection are discussed.

Keywords ToLCNDV · Pathogenicity factor · Phytohormones · AC4 · miRNAs · Host–virus interactions

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Introduction

Phytohormones regulate plant growth and developmental processes through spatial and temporal coordination of gene expression. Perturbation of plant hormone signaling causes developmental defects, some of which are characteristics of viral disease symptoms (Zhu et al. 2005; Padmanabhan et al. 2008). Viral infections were reported to alter the concentration of phytohormones including auxin (Zhu et al. 2005; Padmanabhan et al. 2008; Jin et al. 2016)—an essential phytohormone involved in many facets of plant growth and development (Paque and Weijers 2016). In tryptophan-dependent auxin biosynthesis pathway (Zhao 2014; Mashiguchi et al. 2011), tryptophan aminotransferase-related1 (TAR1) and CYPP450 proteins are involved in the conversion of tryptophan to indole-3-pyruvic acid (IPA) and indole-3-acetaldoxime (IAOx), respectively (Zhao et al. 2002; Stepanova et al. 2008). Auxin-mediated signaling cascade occurs through three components: IAA receptors TIR1/AFB, transcriptional repressors of Aux/IAA and auxin response factor (ARF)—a transcription factor (Ludwig-Muller 2011; Weijers and Wagner 2016; Zhang and Friml 2019). Disruption of auxin signaling during viral infections causes developmental abnormalities that resemble characteristic viral disease symptoms (Padmanabhan et al. 2008; Jin et al. 2016). This modulation in auxin signaling is largely associated with the regulation of host micro-RNAs (miRNAs), viz., miR164, miR167, miR393, miR319 among others. Pivotal roles of host small RNAs, miR164 and miR167, involved in auxin signaling resulting in altered leaf development, organogenesis tissue differentiation, apical dominance and root initiation were documented (Chandler 2016; Li et al. 2016). Expression dynamics of 72 miRNAs in tomato plants inoculated with cucumber mosaic virus (CMV) captured the upregulation of miRNA family genes (miR164, miR165/166 and miR167) at the later stage of CMV infection (Lang et al. 2011). However, mechanistic insights as to how a viral protein interacts with host factors and alters the auxin biosynthesis/auxin signaling and produces symptoms remain elusive.

Geminiviruses (family *Geminiviridae*) are one of the most devastating phytopathogenic viruses worldwide, threatening the global food, nutritional security and sustainability. Geminiviruses are characterized with one (monopartite) or two (bipartite) copies of compact circular single-stranded DNA (ssDNA) genomes each of size 2.6 to 2.8 kb. The viral genome-encoded proteins are multi-functional due to the compact nature of geminivirus genomes. The AC4 protein (in bipartite geminiviruses) and C4 (monopartite homolog) proteins encoded by AC1/C1 ORFs, respectively, are mandatory for viral infection (Carluccio et al. 2018; Chen et al. 2019; Hipp et al. 2016).

In many monopartite geminivirus infections, attenuation of C4 ORF reduces the development of symptoms (Li et al. 2019; Teng et al 2019). Furthermore, when monopartite begomoviruses infect model plants such as *Arabidopsis thaliana* and *Nicotiana benthamiana,* the molecular basis of symptoms development have been attributed to the modulations in cell cycle pathways orchestrated by C4 proteins (Piroux et al. 2007, Mills-Lujan and Deom 2010). Also, C4 proteins alter the intracellular localization of shaggy-like protein kinases (SKη) affecting the brassinosteroid (BR) signaling pathways (Mei et al. 2018a, b). Similarly, the indispensability of AC4 protein in bipartite geminivirus infection has been demonstrated (Hipp et al. 2016; Vinutha et al. 2018; Chen et al. 2019). Previously, we have proven the molecular function of a bipartite begomovirus, ToLCNDV-derived AC4 protein in inducing RNAi suppression (Vinutha et al. 2018). Additionally, over-expression of ToLCNDV-AC4 in tomato (Vinutha et al. 2018) and model plant *Nicotiana benthamiana* has caused severe phenotypic abnormalities. Phenotypic abnormalities such as leaf curling and stunted growth due to AC4 overexpression reminisces the actual ToLCNDV infection in tomato suggesting the pathogenicity determinant role of AC4.

In this context, the molecular basis for disease-associated symptoms in tomato–ToLCNDV interactions is poorly understood. We hypothesize that AC4 protein of ToLCNDV may have a role of symptom determinant that could be partly ascribed to its competence in interacting with multiple host-partners including auxin regulators. In this study, we investigated the tomato host factors that interacted with ToLCNDV-AC4 and found that the proteins involved in auxin/IAA signaling are the crucial components of this interactome. The role of host-derived miRNAs involved in auxin signaling or biosynthesis was also studied. The molecular basis of AC4 and host protein interaction in effecting typical disease symptoms is discussed.

Materials and methods

Tomato genotype, growth conditions and ToLCNDV inoculation

Tomato (*Solanum lycopersicum* var. Pusa Ruby) seedlings of 2–3 leaf stage were maintained in insect-free conditions under 16-/8-h light/dark periods, 18,000 lx, 28–30 °C, and 85% relative humidity at the National Phytotron Facility, ICAR-IARI, New Delhi, India. The seedlings were subjected to agro-inoculation of infectious clones by co-infiltrating the PTR constructs of ToLCNDV DNA-A (TA) and ToLCNDV DNA-B (TB) without any beta satellite components to effect ToLCNDV infection following the previously standardized protocol (Jyothsna et al. 2013).

Total RNA extraction, reverse transcription and preparation of cDNA library

Poly-A RNA fraction of total RNA, extracted from the ToLCNDV-infected tomato leaves at 40 days post-inoculation (dpi), was separated using nucleo-trap mRNA kit (Clontech, USA) according to the manufacturer's protocol. Quality of Poly-A mRNA was assessed on 1.2% agarose gel, and quantification was done using Nanodrop 2000 (Thermo Fisher Scientific, USA). First-strand cDNA was synthesized from the PolyA mRNA (1.0 µg) using SMART MuMLV reverse transcriptase with CDS III Primer following the instructions of Make Your Own "Mate & Plate" Library System (Clontech, USA). The resultant cDNA was converted into dscDNA using LD-PCR kit (Clontech, USA) (Clontech, USA) and the dscDNA (> 200 bp) was further purified using CHROMA SPIN TE-400 columns (Clontech, USA). The dsDNA (3 µg), and linearized prey library vector pGADT7-Rec (6 µg), was co-transformed into yeast competent cell Y187 following the library-scale transformation protocol (Yeast Transformation System 2 Manual, Clontech, USA). Transformation efficiency was estimated in SD/Leu media. After incubation at 30 °C for 4 days, all the colonies were harvested in a freezing medium (YPDA in 25% glycerol) to form an Y2H library and were stored at – 80 °C as aliquots of 1 mL and the quality of the cDNA library was ascertained.

PCR amplification of prey vector inserts from yeast cells

The successful ligation of cDNA fragments into the prey vector, pGADT7-Rec (Clontech, USA), was ascertained by performing PCR directly using the yeast colonies and matchmaker Insert check PCR Mix 2 (Clontech, USA). The thermal cycling conditions followed were 94 °C for 1 min followed by 30 cycles of 98 °C for 10 s and 68 °C for 3 min.

Bait plasmid construction

The full-length coding sequence of ToLCNDV-AC4 was PCR amplified using high-fidelity Phusion *Taq* DNA polymerase (New England Biolabs, Beverly, MA, USA) and the primers used were

AC4-*EcoR*1 F (5′GATGAATTCATGGGTCTCCGCATATCCAT3′)/AC4-*Pst*1 R (5′GACGTCCCCATGGGTCTCCGCATATCCAT3′) and AC4-*BamH*I R (5′GCTGGATCCCTAGAACGTCTCCATCTTTGT3′). pGBKT7 (BD) and pGADT7 (AD) vectors were digested with *EcoR*I/*Pst*I and *EcoR*I/*BamH*I, respectively (New England Biolabs, Beverly, MA, USA) and their corresponding inserts were ligated.

Yeast two-hybrid assay

To screen yeast two-hybrid (Y2H) library using ToLCNDV-AC4 as a bait protein, the yeast strain Y2H Gold harboring pGBKT7-AC4 insert was grown in 50 mL SD/-Trp at 30 °C with 180 rpm until the OD_{600} reaches 0.8 (16–20 h). The cells were harvested by centrifugation (1000 g for 5 min) and cell density was adjusted to $> 1 \times 10^8$ cells/mL in SD/-Trp. Five mL of the bait strain cell culture and 1 mL of the library strain Y187 cell culture (two-hybrid library) were added to 45 mL of 2 × YPDA containing 50 µg/mL kanamycin and incubated at 30 °C at 50 rpm, until zygotes appear, which were microscopically examined (Olympus, India) after 20–24 h. Cells were harvested by centrifugation at 1000 g for 5 min, the cell pellet was washed with 0.5 × YPDA containing 50 µg/mL kanamycin and finally resuspended in 10 mL of 0.5 × YPDA containing 50 µg/mL kanamycin. To calculate the mating efficiency, 100 µL of the mated culture (1/10, 1/100, 1/1000 and 1/10,000 dilutions) was spread on to SD/-Trp, SD/-Leu, and SD/-Trp/-Leu agar plates and incubated at 30 °C for 3–5 days. The remaining culture was plated on to SD/-Trp/-Leu/X-a-Gal/AbA (40 µg/mL X-a-Gal and 200 ng/mL Aureobasidin A) agar plates. In total, 30 plates (110 mm), with 200 µL cells per plate were plated and incubated at 30 °C for 3–5 days. The blue colonies that appeared on SD/-Trp/-Leu/X-a-Gal/AbA agar plates were streaked on high stringency plates SD/-Ade/-His/-Trp/-Leu/X-a-Gal and incubated at 30 °C for 3–5 days. The AD plasmids were isolated from blue colonies and inserts characterized by sequencing. For the control experiments, yeast strain Y2H Gold containing plasmids pGBKT7-53 and pGADT7-T (positive control); pGBKT7-lam and pGADT7-T (negative control) were grown on SD/-Trp/-Leu media for 24 h and plated on to SD/-Trp/-Leu agar media and SD/-Ade/-His/-Trp/-Leu/X-a-Gal agar media and incubated at 30 °C for 3–5 days. A preliminary Y2H assay was performed by co-transforming pGBKT7-ToLCNDV-AC4 with pGADT7 devoid of cDNA insert on QDO plates to assess any false positives. An auto-activation test was also performed for pGBKT7-ToLCNDV-AC4 on SDO plate to ensure that AC4 protein does not autonomously activate the reporter genes in Y2HGold strain of yeast in the absence of a prey protein.

Tomato transformation and regeneration

Explants derived from healthy, young tomato leaves were regenerated and co-cultivated with *Agrobacterium* carrying AC4 constructs and without the gene constructs as described by Praveen et al. (2010). Explants were incubated in Petri plates on callus medium containing MS salts, vitamins, 3% sucrose, 0.2 mg/L NAA, 1 mg/L BAP, 50 mg/L kanamycin (incase of explants carrying AC4 construct) and 200 mg/L

cefotaxime (pH 5.8). The culture conditions were 25 °C for a 16-h photoperiod. They were later transferred to shooting and rooting medium, respectively (MS salts, 2.5 mg/L BAP, 0.5 mg/L IBA; and half MS, 0.2 mg/L NAA, respectively). Kanamycin (50–100 mg/L) was used for selection of transgenic tomato plants. The rooted explants were transplanted into small pots containing sterilized soil mix (soil:peat:vermiculite, 1:1:1) for hardening of plants.

Exogenous application of IAA and documentation of phenotypic data

The response of ToLCNDV-infected tomato plants were examined following exogenous application of IAA at 40 dpi. ToLCNDV-infected plants and healthy plants (8–12 nos) of similar stature from their respective plant population were chosen and divided into three groups. One among them from the infected and healthy plants served as a control. The plants were sprayed 25 mL of IAA (50 mg/L) or water once a week and the treatment were repeated twice at 2nd week and 3rd week. Plant height was measured at 15 days after the third spray. Healthy tomato seedlings at the same stage sprayed with water served as controls.

Quantification of auxin levels

The auxin extracts were isolated from healthy, ToLCNDV-infected and AC4 transgenic tomato leaves using 10% aqueous methanol solution. The sample extracts were purified using HLB columns (30 mg/mL, Waters). A solution of stable isotope-labeled standard: $^{13}C_6$IAA (8/mol) was added to the purified samples. The sample analyte (auxin) was eluted using 80% methanol and then analyzed by LC–MS/MS method (Floková et al. 2014).

Quantification of host miRNAs

Small RNAs were extracted from 100 mg of leaf tissues using mirVana®kit (Ambion). Small RNA fraction was polyadenylated using *E. coli* poly-(A) polymerase (NEB) and the poly-(A) tailed small RNA was purified using a cartridge supplied with mirVana Probe and Marker Kit (Ambion). Small RNA cDNA (srcDNA) was generated by mixing 500 ng of poly-(A) tailed RNA and 1 µg of extension tail primer and 20 U of reverse transcriptase (M-MuLV Reverse transcriptase NEB), 1 µL dNTP mix (10 mM) in a 40 µL reaction volume at 37 °C for 60 min. Reverse transcriptase was inactivated by incubating at 70 °C for 15 min which was followed by addition of 5U RNase H (NEB) to remove small RNAs. The quantitative real-time PCR (qRT-PCR) was performed using 2X Light CyclerR 480 SYBR Green 1 master mix (Roche) in the Light CyclerR 480 II (Roche), and the PCR conditions were as follows: 94 °C for 10 min, then 30 cycles of 94 °C for 15 s, 55 °C for 30 s. and 72 °C for 30 s. The quantitative real-time PCR (qRT-PCR) experiment was conducted in triplicate and the relative expression level of all the miRNAs were calculated with tomato U6 small nuclear RNA (U6snRNA) as a reference gene following the comparative $2^{-\Delta\Delta Ct}$ method.

Quantification of target transcripts and transcription factors (TFs)

First-strand cDNA was synthesized from 1 µg of Dnase-treated total RNA from infected and healthy tomato leaves (10, 15 and 25, 40 dpi) using first-strand cDNA synthesis kit (Qiagen) according to the manufacturer's instructions. RT-qPCR reaction mixture (25 µL) consisted of 25 ng of cDNA, 0.5 mM specific forward (F) and reverse (R) primer each and 1X SYBR Green (Roche). The transcripts AC4, LoxD [ATGGCACTTGCTAAAGAAATT (F), TCATATCGATACACTATTTGG (R)], TCP1 [TCCCATTCGGCGGCGACCTA (F), TGATCCGTACCTCTGGTATTGTGGCA(R)] and ARF8 [TGGGAAAGGAAGAGGCTGAA (F), GCGATCCAAGAGATGGCATT (R)] were quantified and their relative expression levels were calculated using the tomato *actin* gene as a reference following the comparative $2^{-\Delta\Delta Ct}$ method. Tomato *actin* gene was found to be one of the most suitable genes for studying virus–host interactions (Praveen et al. 2010; Wieczorek et al. 2013).

Detection of miRNAs

Twenty microgram of RNA was resolved in 17% polyacrylamide gel (a 19:1 ratio of acrylamide to bis-acrylamide, 7 M urea and 20 mM MOPS/NaOH buffer, pH 7.0). Following the electrophoresis, the RNA was transferred to positively charged nylon membrane (Millipore) at a constant 20 V for 30–60 min at 4 °C by electroblotting and UV cross-linking was done. Hybridization, washing and detection were performed using ssDNA oligonucleotide probes complementary to tomato miRNAs, viz., miR164, miR167, miR393 and miR319 according to the manufacturer's instructions.

Results

Symptoms of ToLCNDV-infected tomato and viral titre

Phenotyping of agro-inoculated tomato seedlings for ToLCNDV-specific symptoms revealed slight puckering of leaves at 5 dpi. Initially, ToLCNDV-inoculated plants showed little visual symptoms (disease severity scale—0). However, with the progression of time, symptoms ranged from slight leaf puckering (scale—2) through leaf curling (scale—6), arrest

of plant growth, severe leaf curling, yellowing and deformed leaves (scale—10) at 30 dpi (Fig. 1a). The disease-associated symptoms commensurate with the viral copy number in the infected plants. Virus accumulation kinetics, ascertained by quantifying AV3 transcript, revealed that viral copy number in the infected leaves showed a progressive increase from 8.54×10^5 (8 dpi) to 1.26×107^5 (40 dpi) (Fig. 1b). Symptoms severity kinetics was studied by plotting the various symptoms on a scale of 0–10 against the dpi. The severity progressed from early appearance of leaf curling through leaf puckering and culminating in stunted growth of the seedlings (Fig. 1c).

Ectopic expression of ToLCNDV-AC4 protein modulates tomato phenotype

Gene construct harboring ToLCNDV-derived *AC4* gene previously developed in the binary plasmid pCAMBIA 2301 was used for tomato transformation studies (Vinutha et al. 2018). Transgenic tomato lines constitutively expressing the ToLCNDV*AC4* gene (ToLCNDV-AC4-OE) were developed by transforming and regenerating tomato plants of variety Pusa Ruby (PRu). Overexpression of the virus-derived *AC4*gene in a tomato genetic background showed severe morphological abnormalities such as stunted and aberrant growth, reduced leaf lamina, typical leaf curl symptoms and abnormal root characteristics (Fig. 2).

ToLCNDV-AC4 interacts with tomato proteins involved in auxin metabolism

Yeast two-hybrid (Y2H) screening of tomato cDNA library (prey plasmids) using ToLCNDV-AC4 as a bait protein identified six positive colonies on SD/Leu-/Trp-/His-/Ade-plates/X-gal media (Table 1). Sequence analysis of the plasmid inserts from the positive colonies revealed that two colonies had the inserts that encode cytochrome P450 monooxygenase, four colony each had inserts that encode tryptophan amino transferase (TAR1) like protein, senescence-associated gene (SAG) protein, TOi54 and an unknown protein (Table 2; Fig. 3a, b). Furthermore, interaction of these proteins with ToLCNDV-AC4 was reconfirmed by co-transforming the corresponding cDNA inserts in AD with BD-AC4 plasmids into yeast cells and revalidated on QDO/X-gal medium (Fig. 3c). Thus, the bona fide

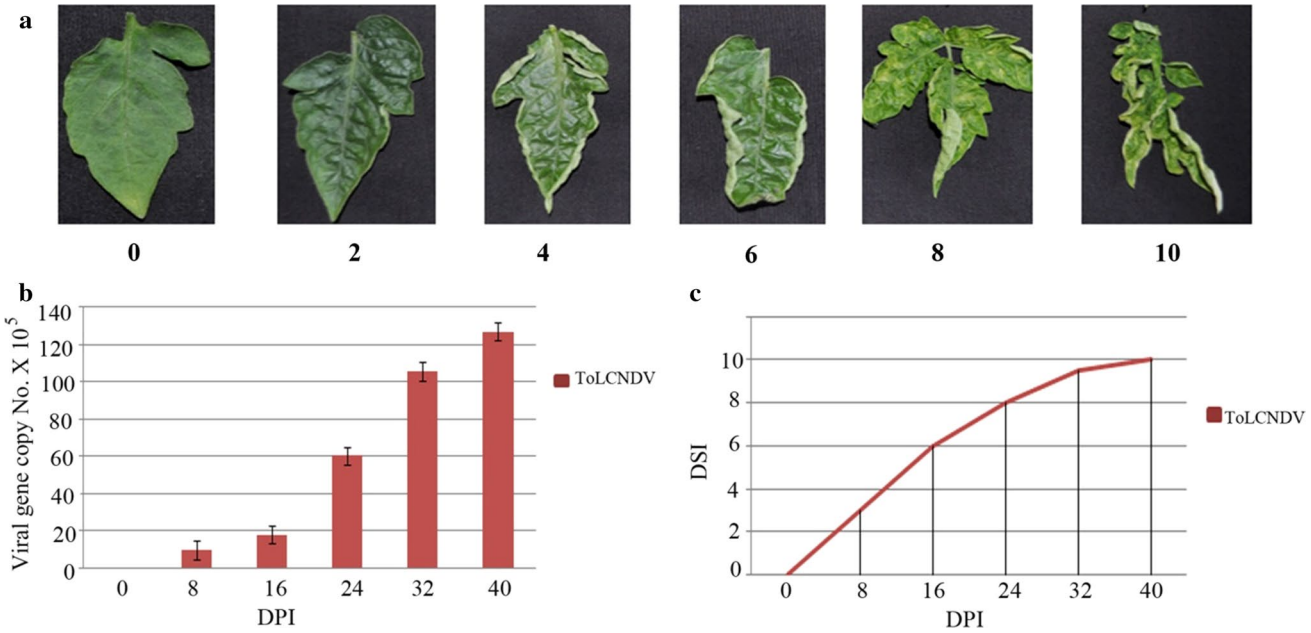


Fig. 1 Phenotypic symptoms of tomato following *Tomato leaf curl New Delhi virus* infection. **a** The symptoms were categorized into a 10-point disease severity index or scale (0: no symptoms, 2: slight puckering of leaf, 4: curling of leaf edge and moderate puckering, 6: severe curling of leaf, 8: slight yellowing of leaf with severe curling and cup shaped leaf, 10: stunted plant growth with very severe curling, yellowing and deformation of leaf). Agro-inoculation-based ToLCNDV infection was performed in a 20 days old tomato seedlings (variety Pusa Ruby-PRu) using the *Agrobacterium* cultures harboring DNA-A, DNA-B and β satellite genetic components of the virus in binary vector. Symptoms were recorded at an interval of 8 days till the appearance of severe symptoms associated with the disease. **b** Virus accumulation kinetics in ToLCNDV-infected tomato leaves. Virus titre was estimated at different time intervals post-viral inoculation by quantifying the expression of AV3 transcript in infected plants and compared that to endogenous actin transcript. Error bars represent standard deviation of mean data in three replications. **c** Disease severity index following ToLCNDV infection in tomato. The scoring of disease symptoms following viral infection was performed based on the disease severity scale and DSI (disease severity index) was estimated

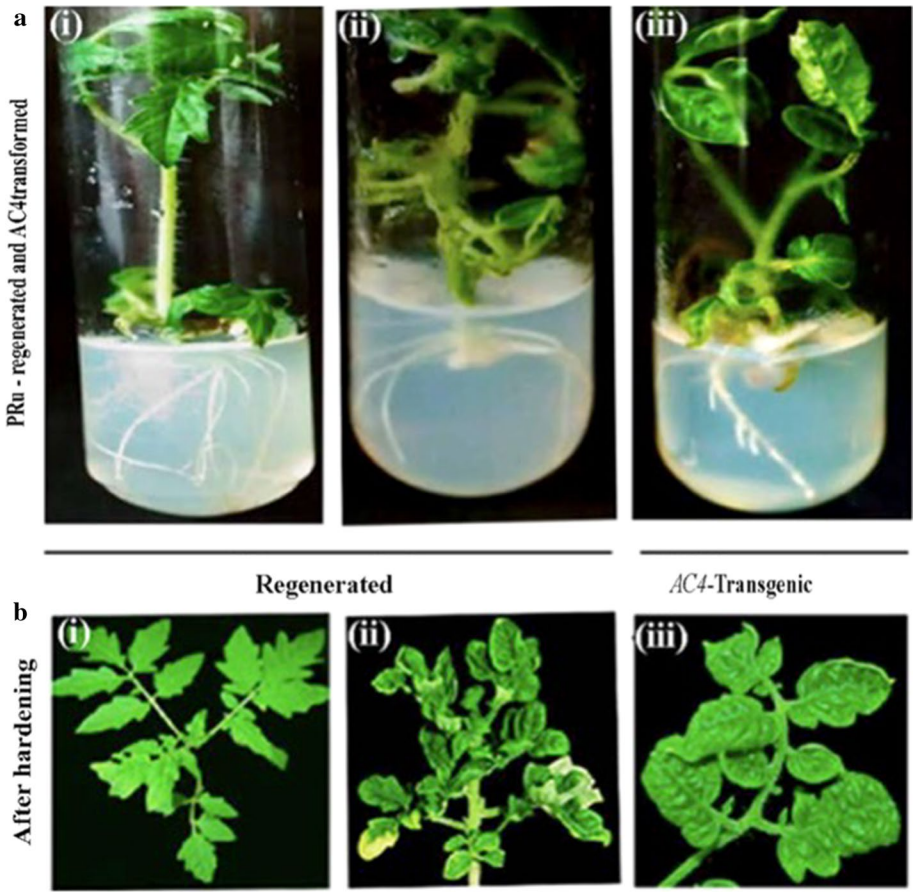


Fig. 2 Root and leaf morphological changes due to AC4 expression in plants [variety: Pusa Ruby (PRu)]. **a** Regenerated healthy, ToLCNDV-infected plants and AC4-transgenics. (i) Healthy tomato leaf explants inoculated with empty vector grown on MS medium showing normal leaf and root growth. (ii) ToLCNDV-infected tomato leaf explants grown on MS medium showing lesser root growth and curling of leaves. (iii) 35S:ToLCNDV-AC4-overexpressing tomato plants showing poor root growth, stunting and curling of leaves. **b** Regenerated and AC4 transgenic plants 2 months after hardening showing normal leaf growth (i), leaf curling symptoms in ToLCNDV-infected plants (ii) and in 35S:ToLCNDV-AC4-overexpressing plants (iii)

Table 1 Parameters of Y2H screening library quality

Parameters	Values
Transformation efficiency	3.22×10^5 cfu/µg AD vector
Total independent colonies	6.39×10^5 cfu/library
Library concentration	1.4×10^7 cells/mL
Minimum length	0.2 kb
Maximum length	1.8 kb
Average length	0.85 kb

interactions of these host proteins with ToLCNDV-derived AC4 in yeast cells were confirmed.

ToLCNDV infection reduces auxin content and disrupts its signaling in tomato

ToLCNDV-infected leaves showed a 58.2% reduction (7.6 ng/g FW) in endogenous auxin (IAA) content in comparison to the healthy plants (18.2 ng/g FW) (Fig. 5b). Some of the symptoms caused by ToLCNDV mimic the phenotypic aberrations associated with auxin-deficient mutants or plants often resemble mutants with the compromised auxin biosynthesis/or signaling pathways. To

Table 2 ToLCNDV-AC4 and unique host protein interactors identified from the Y2H screen

Sl. no.	GenBank accession	Frequency of occurrence	Gene homolog
1	Solyc00g068970.2	5	Cytochrome P450 like_TBP
2	Solyc00g006680.1	3	Senescence-associated protein (SAG)
3	Solyc07g039290.1	1	TO54-2
4	Solyc00g009760.2	6	Cytochrome P450 monooxygenase
5	Solyc05g025570.1	2	Unknown protein
6	XM_015222884	5	TAR1-like

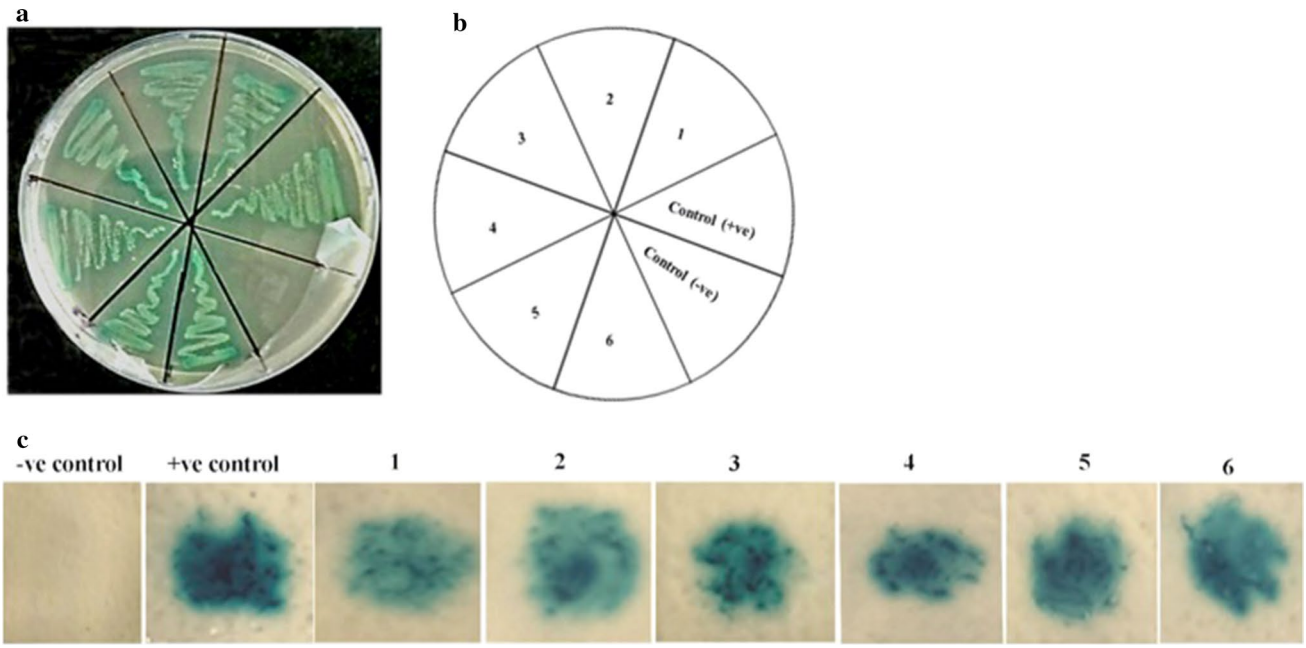


Fig. 3 Interaction of ToLCNDV-AC4 with tomato leaf cDNA libraries through yeast two-hybrid assay. **a** Representation of positive interactions of ToLCNDV-AC4 with host proteins along with controls plated on SD-Ade/-his/-Leu/-Trp/X-Gal media. **b** ToLCNDV-AC4 showed interaction with the following host proteins: (1) cytochrome P450 like_TBP. (2) SAG. (3) TO54-2. (4) Cytochrome P450 mono-oxygenase. (5) TAR1-like. (6) Unknown protein, positive control-pGBKT7-53 + pGADT7-T; negative control: pGBKT7-lam + pGADT7-T. **c** The reconfirmation of ToLCNDV-AC4 and host protein interactions: X-Gal arrays for retransformed Y2H cells on SD-Ade/-his/-Leu/-Trp/X-Gal media. 1: Negative control:Y2H gold [pGBKT7-lam] + Y187[pGADT7-T]. 2: Positive control: Y2H Gold [pGBKT7-53] + Y187 [pGADT7-T]; 3: Y2H Gold [[pGBKT7-ToLCNDV-AC4] + Y187 [pGADT7-CytP450 like TBP]; 4: Y2H Gold [pGBKT7-ToLCNDV-AC4] + Y187 [pGADT7-SAG]; 5: Y2H Gold [pGBKT7-ToLCNDV-AC4] + Y187 [pGADT7-TO54-2]; 6: Y2H Gold [pGBKT7-ToLCNDV-AC4] + Y187 [pGADT7-CytP450 monooxygenase]; 7: Y2H Gold [pGBKT7-ToLCNDV-AC4] + Y187 [pGADT7-TAR1 like]

establish if auxin signaling pathway is certainly compromised by ToLCNDV infection, qRT-PCR was carried out to record the changes in the expression levels of those miRNAs that are involved in auxin signaling and leaf morphogenesis. ToLCNDV infection caused significant changes ($P < 0.05$) in the expression levels of miR164, miR167, miR393, miR319 (Fig. 4a). Approximately 14-fold increase in the expression of miR164 was observed in the infected plants followed by miR393 (10.9-fold), miR167 (9-fold) and miR319 (7.7-fold), respectively (Fig. 4b). Corroborating the miRNA expression, significant downregulation of target transcripts of respective miRNAs such as *ARF*8 (miR167), *TCP*1 (miR319) (< 0.5-fold) and *Lox*D transcripts (miR393) (0.7-fold) was observed (Fig. 4b). Significant downregulation ($P < 0.05$) of the genes *slTAR*1, *slCYP*450 monooxygenase involved in IAA biosynthesis and *slSAG* (< twofold) involved in host auxin signaling processes were also documented during ToLCNDV infection (Fig. 4c).

Exogenous application of IAA restores healthy phenotype

To further examine the effect of auxin on ToLCNDV infection, tomato plants were sprayed with auxin analogue IAA (50 mg/L). As shown in Fig. 5a, healthy tomato plants grew taller and had more leaves compared to the virus-infected plants devoid of IAA spray. ToLCNDV-infected tomato plants with characteristic leaf curling and stunted growth exhibited remission of the symptoms and healthy phenotypes of the plants were recovered following the IAA spray. Also, the IAA content of tomato plants following exogenous application was on par with that of control healthy plants (Fig. 5b). Further significant decrease ($P < 0.5$) in the expression of auxin signaling miRNAs, viz., miR393, miR164, miR167 and miR319 (Fig. 5d) was observed in IAA-treated infected plants compared to the non-treated infected plants. Thus, it appears that ToLCNDV-AC4 hijacks host proteins, viz., TAR1, CYTP450 monooxygenase that are involved in IAA biosynthesis to

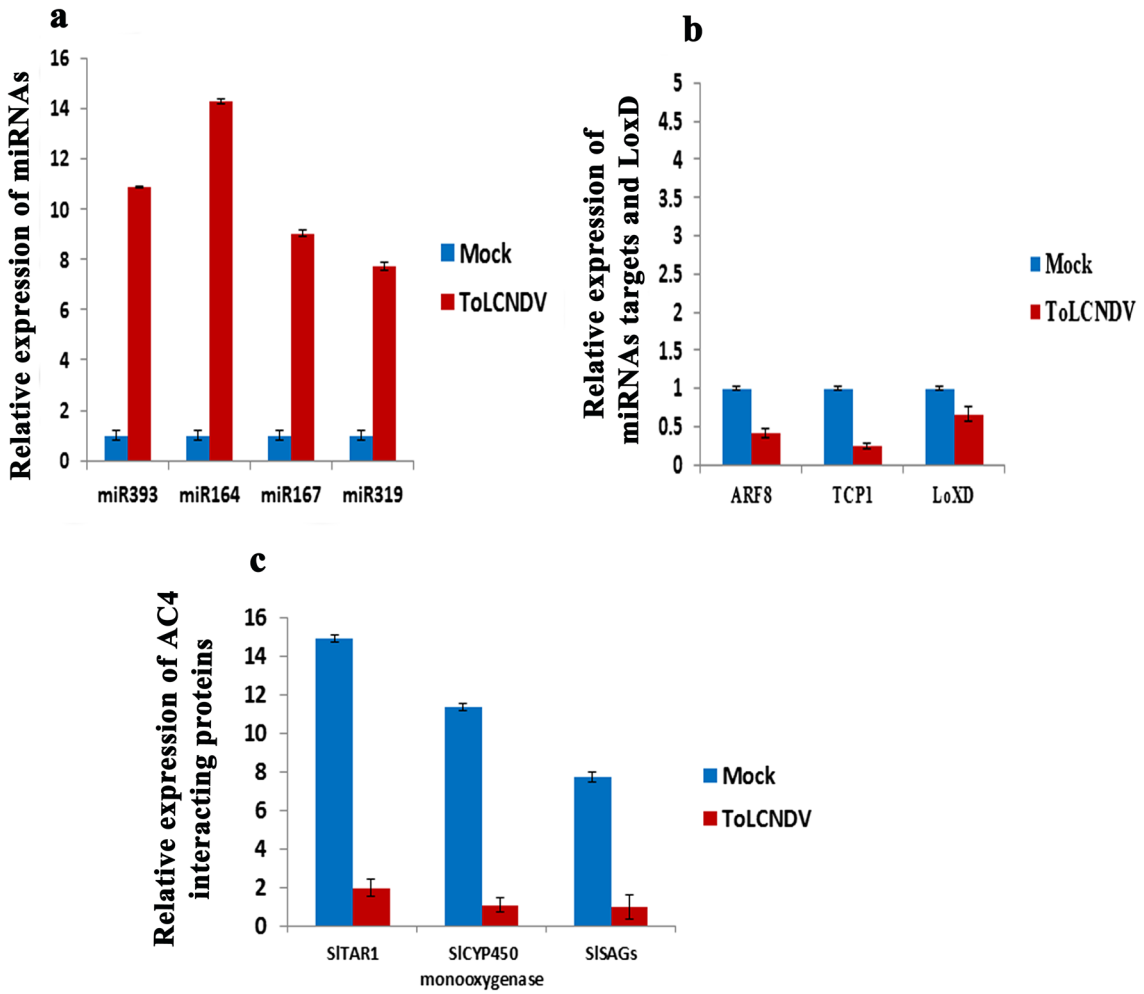


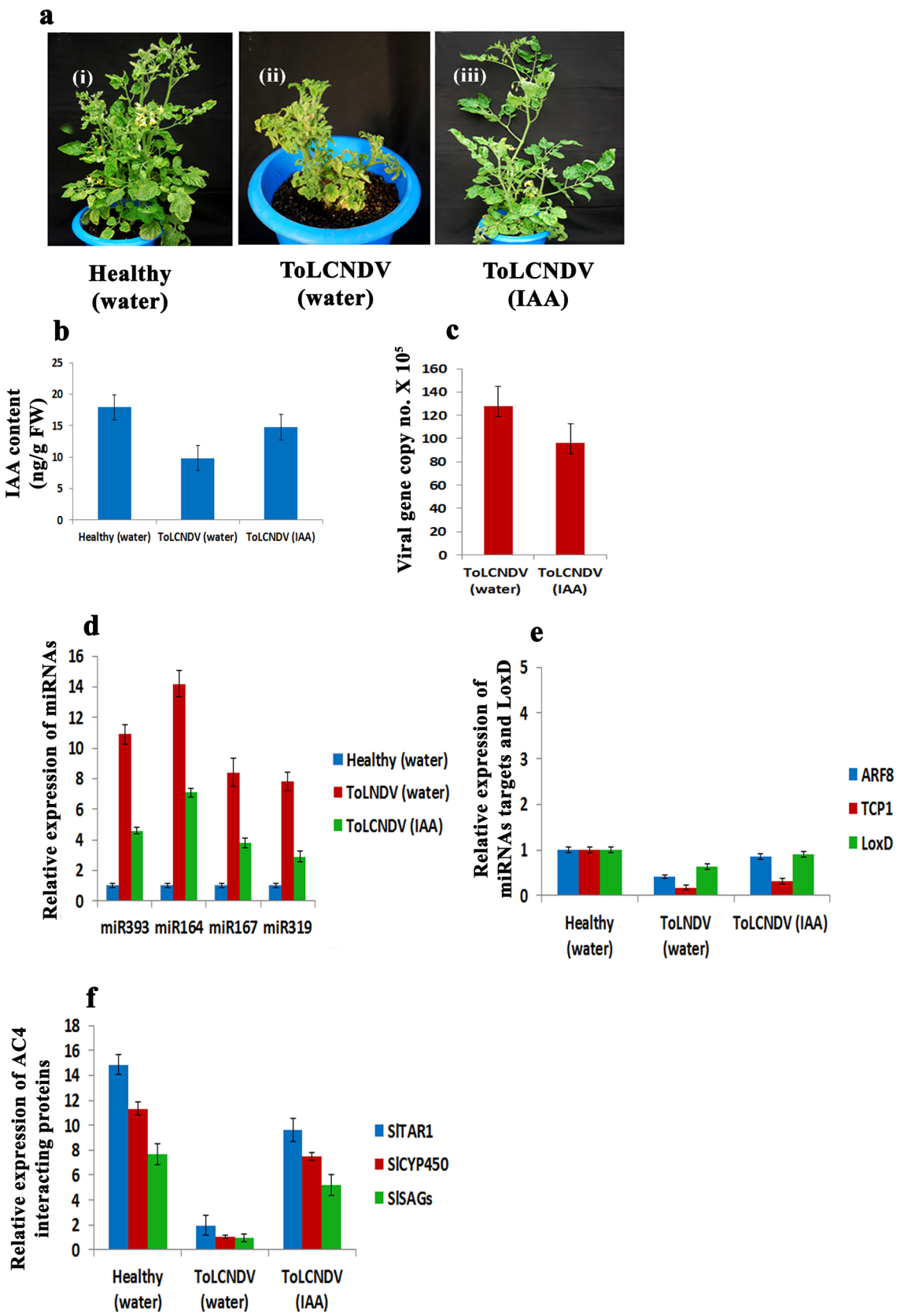
Fig. 4 Effects of ToLCNDV infection on the expression of miRNAs, miRNA targets, LoxD and auxin-responsive proteins. **a** The relative expression levels of miRNAs in mock- and ToLCNDV-infected tomato plants. The expression levels were normalized using U6 RNA. **b** The relative expression levels of ARF8, TCP1 and LoxD genes in mock- and ToLCNDV-infected tomato plants. **c** The relative gene expression of AC4-interacting proteins in mock- and ToLCNDV-infected plants. The expression levels were normalized using actin gene (for transcripts) and U6RNA (for miRNAs) gene expression. The average values from three biological replicates of qRT-PCR are shown

decrease endogenous IAA levels that subsequently leads to the development of symptoms.

Overexpression of ToLCNDV-AC4 recapitulates features of ToLCNDV infection

Tomato transgenic plants overexpressing ToLCNDV-AC4 were analyzed for the expression status of selected miRNAs, their respective target mRNAs and auxin content. Expression of miR164, miR167, miR319 and miR393 and their corresponding target transcripts, viz., *ARF*8, *TCP*1 and *Lox*D followed a pattern comparable to that of ToLCNDV-infected plants. Expression of miRNAs, namely miR164 (9.4-fold) and miR393 (5.5-fold), was significantly ($P < 0.05$) altered; however, overexpression of AC4 had relatively less pronounced effect on the expression levels of miR167 (2.8-fold)

Fig. 5 Restoration of the leaf curling and stunted growth phenotype of ▶ ToLCNDV-infected tomato plants. Plant images were taken after 40 days of exogenous application of IAA (50 mg/L) application. **a** (i) Healthy tomato plants after exogenous water application; (ii) ToLCNDV-infected tomato plants after exogenous water application. (iii) Restored phenotype of ToLCNDV-infected tomato plants after IAA application. **b** IAA content in healthy, ToLCNDV-infected (water sprayed) and ToLCNDV-infected (IAA sprayed) plants after 40 days of exogenous IAA application. **c** Virus titre was estimated by quantifying the expression of AV3 transcript in infected and IAA-treated plants and compared to that of endogenous actin transcript. Error bars represent standard deviation of mean data in three replications. **d** The relative expression levels of miRNAs in mock-, ToLCNDV-infected tomato plants and IAA-treated infected plants. The expression levels were normalized using U6 RNA. **e** The relative expression levels of ARF8, TCP1 and LoxD genes in mock-, ToLCNDV-infected and IAA-treated infected tomato plants. **f** The relative gene expression of AC4-interacting proteins in mock-, ToLCNDV-infected and IAA-treated infected tomato plants. The expression levels were normalized using actin gene (for transcripts) and U6RNA (for miRNAs) gene expression. The average values from three biological replicates of qRT-PCR are shown


a
(i)
(ii)
(iii)
Healthy (water)
ToLCNDV (water)
ToLCNDV (IAA)
b
IAA content (ng/g FW)
25
20
15
10
5
0
Healthy (water)
ToLCNDV (water)
ToLCNDV (IAA)
c
Viral gene copy no. X 10^5
160
140
120
100
80
60
40
20
0
ToLCNDV (water)
ToLCNDV (IAA)
d
Relative expression of miRNAs
16
14
12
10
8
6
4
2
0
miR393
miR164
miR167
miR319
Healthy (water)
ToLNDV (water)
ToLCNDV (IAA)
e
Relative expression of miRNAs targets and LoxD
5
4
3
2
1
0
Healthy (water)
ToLNDV (water)
ToLCNDV (IAA)
ARF8
TCP1
LoxD
f
Relative expression of AC4 interacting proteins
18
16
14
12
10
8
6
4
2
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Healthy (water)
ToLCNDV (water)
ToLCNDV (IAA)
SlTAR1
SlCYP450
SlSAGs

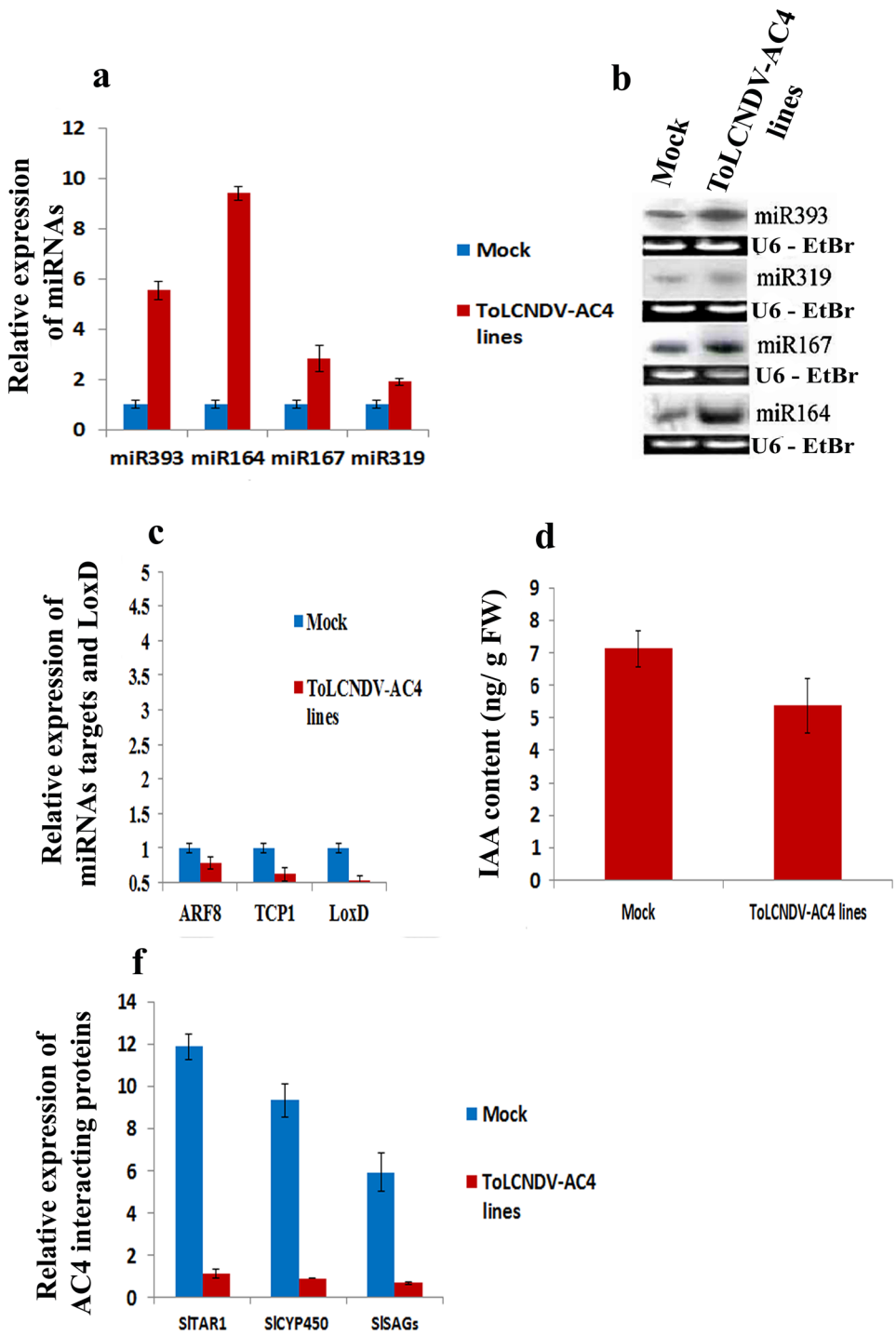

Fig. 6 ToLCNDV-AC4 overexpression in tomato recapitulates the small RNA transcriptome features of ToLCNDV infection. **a**, **b** The relative expression of miRNAs in transgenic lines analyzed by qRT-PCR and Northern blotting. **c** The relative expression ARF8, TCP1 and LoxD in transgenic lines analyzed by qRT-PCR. **d** The relative IAA content in leaves of mock-inoculated and TOLCNDV-AC4 tomato lines. **e** The relative gene expression of AC4-interacting proteins in mock and ToLCNDV-AC4 lines. Expression levels were normalized using actin gene (for target transcripts) and U6RNA (for miRNAs), respectively. The mean values from three biological replicates of qRT-PCR are shown

and miR319 (1.9-fold). Northern blot hybridization detected the expression of selected host miRNAs (Fig. 6a, b). Analysis of target transcripts, namely *ARF*8, *TCP*1 and *Lox*D in ToLCNDV-AC4 lines showed < 0.8-fold changes in their expression compared to that of mock-inoculated plants (Fig. 6c). Furthermore, the relatively low expression levels of target mRNAs commensurate with the significantly ($P < 0.5$) lower levels of auxin content in ToLCNDV-AC4 transgenic lines (5.37 ng/g of FW) compared to the mock-inoculated plants (7.13 ng/g of FW) (Fig. 6d). However, exogenous application of auxin (50 mg/L) on tomato plants overexpressing AC4 exhibited little recovery from the symptoms; this could be due to significantly high levels of AC4 transcripts in transgenic lines compared to ToLCNDV-infected plants (Vinutha et al. 2018).

Discussion

Geminiviruses encode multi-functional proteins that modulate the host-plant gene expression to create favorable condition in vivo for the proliferation of viral particles. Geminiviruses effectively utilize their compact genomes (either single or two copies of ~ 2.7 Kb ssDNA genomes) to encode versatile proteins based on the principle of cellular economy. Previously, we have shown that ToLCNDV-encoded AC4 protein is a potent viral suppressor of RNA silencing (VSR) that effectively blocks the viral DNA methylation by sequestering host AGO4 and reverses transcriptional gene silencing (TGS) (Vinutha et al. 2018). Although transient agro-patch assays disclosed that AC4 is also a potent suppressor of post-transcriptional gene silencing (PTGS), host factors associated with the reversion of RNA silencing remain elusive. On the other hand, ectopic expression of AC4 in tomato resulted in severe phenotypic abnormalities that typically mimic the ToLCNDV infection traits (Ramesh et al. 2007; Praveen et al. 2010; Vinutha et al. 2018). Additionally, some of the symptoms caused by ToLCNDV reminisces the phenotypic aberrations associated with auxin-deficient mutants or mutants characterized with compromised auxin biosynthesis or signaling (Cheng et al. 2006; Benjamins and Scheres 2008; Wu et al. 2015). AC4 is a micro-protein; hence, it is highly plausible for it to have a role in the development of symptoms through interacting with host components. This premise led us to explore the role of AC4 in modulating the tomato proteins involved in auxin biosynthesis or signaling.

The protein C4, a functional homolog of AC4, encoded by monopartite geminiviruses interacts with a group of SHAGGY-like kinases affecting brassinosteroid pathway (Piroux et al. 2007), enhances cell division and induces characteristic symptoms (Mills-Lujan and Deom 2010).

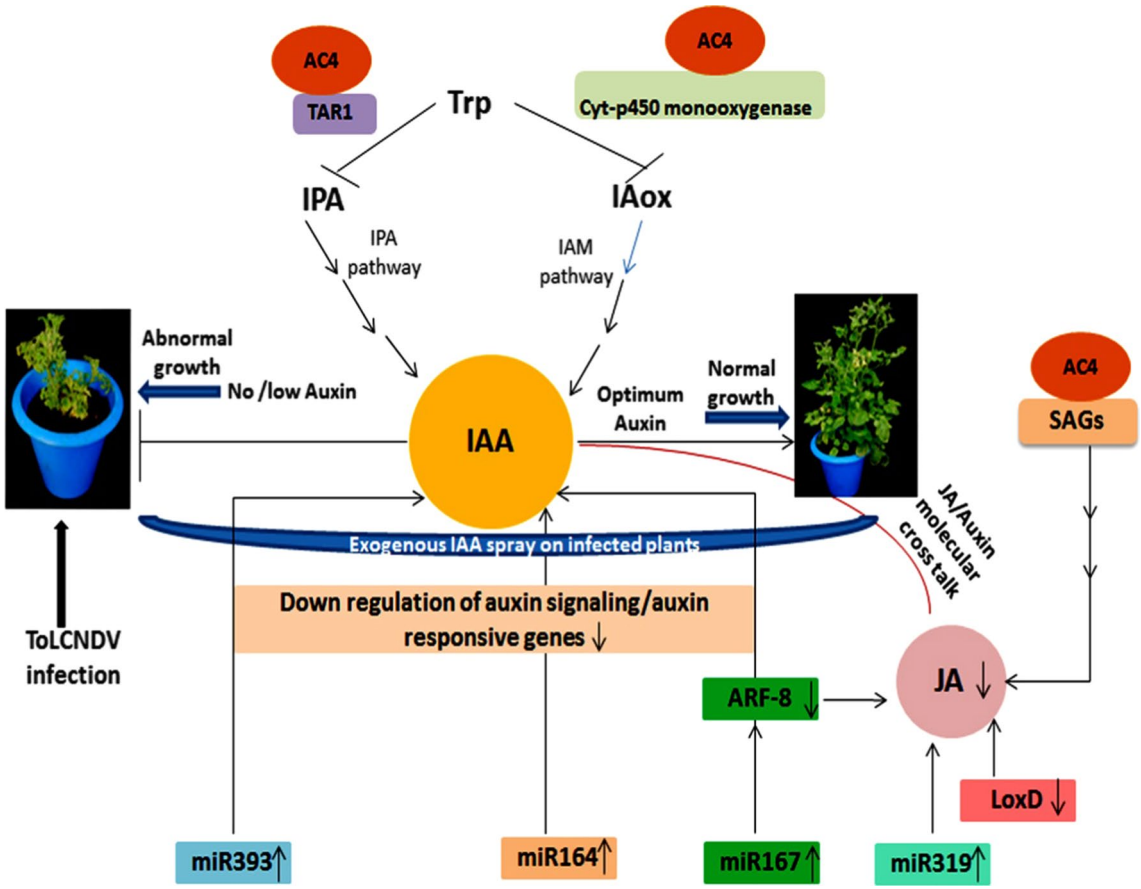

Fig. 7 Molecular mechanism of ToLCNDV infection modulating the auxin homeostasis in tomato. Induction of tomato miR319 by ToLCNDV infection and the interaction of AC4 with SAGs suppressed JA-mediated defense response to facilitate the virus infection and appearance of typical symptoms. Inhibition of JA induced defense pathway was further confirmed by low expression levels of LoxD. Induction of miR393, miR164 and miR167 expression led to the downregulation of auxin signaling or auxin-responsive genes, affecting the auxin homeostasis. AC4 interacts with cyt-p450 monooxygenase or cyt-450 family proteins—which utilize Trp as a precursor to synthesize indoleacetaldoxime—the precursor of IAA through IAM pathway. Similarly, AC4 interacts with TAR1 which mediates the conversion of Trp to IPA (indole-3-pyruvic acid)—the precursor of IAA through IPA pathway. Exogenous application of auxin on the plants remitted the typical symptoms and showed phenotypic recovery

Characteristically, C4/AC4 induces hyperplasia and tumorigenic growth of the infected plant tissues likening it to a viral 'oncogene' (Fondong 2019). Moreover, interaction between C4 and transmembrane proteins has been proposed to elucidate the observed symptoms (Zeng et al. 2018). Consistent with this, Tomato yellow leaf curl virus (TYLCV)-encoded C4 has been shown to interact with both BAM1 and BAM2 (BARELY ANY MERISTEM1 and 2), the receptor-like kinases (RLKs) (Rosas-Diaz et al. 2018). Similarly, Beet curly top virus-encoded C4 also interacts with an RLK, CLV1 (CLAVATA 1), affecting shoot development (Li et al. 2019). Thus, analyzing the commonalities of interactions of AC4/C4 and their host factors divulge that AC4/C4 endeavors to attenuate the RNAi spread and promotes the cell division.

Our investigation provides yet another functional dimension to ToLCNDV-AC4 that interacts with host factors such as TAR1-like protein and CYP450 monooxygenase and attenuates host auxin biosynthesis consequently inducing characteristic symptoms. Instances of perturbation of auxin/IAA signaling during viral infections have been reported (Zhu et al. 2005; Padmanabhan et al. 2008; Jin et al. 2016; Zhang and Friml 2019). Rice dwarf virus-encoded RDV-P2 binds the host OsIAA10 repressor protein of IAA and blocks its degradation through proteasome pathway. Also, the interaction of RDV-P2–OsIAA10 complex with auxin response factors (ARFs) inhibits auxin signaling causing the development of symptoms (Jin et al. 2016). Rice black streak dwarf virus (RBSDV), infection affects auxin homeostasis and represses the auxin pathway genes (Zhang and Friml 2019). Auxin has long been recognized as a regulator of defense response as it activates defense responsive genes and imparts resistance (Rosas-Diaz et al. 2018). Previous reports have shown that, *Arabidopsis* mutants defective in auxin pathway are more susceptible to *Alternaria brassicicola* (Qi et al. 2012) suggesting the pre-dominance of auxin in plant defense mechanisms. The activity of viral suppressors of RNA silencing has been linked to the suppression of auxin/IAA signaling and symptoms development (Kasschau et al. 2003) concomitant with the upregulation of miRNAs targeting auxin-responsive factors (ARFs) (Islam et al. 2018). The pattern of expression of miRNAs, miR164 and miR167, associated with the disruption of auxin/IAA signaling from this study is consistent with the earlier reports (Tagami et al. 2007; Bazzini et al. 2007). Furthermore, host miRNAs; miR164 and miR167, involved in auxin signaling play pivotal roles in plant growth and developmental activities such as organogenesis, leaf morphogenesis, vascular tissue differentiation, apical dominance and root initiation (Chandler 2016; Li et al. 2016; Koyama et al. 2010).

On the other hand, miR319 and its target genes are not only the key regulators of leaf morphogenesis (Martín-Trilloand Cubas 2010; Lopez et al. 2015) but also affect the JA biosynthesis defense pathway. It was proposed that induction of JA-mediated defense responses could be an integral part of auxin-mediated defense signaling pathway in rice thus, underscoring the importance of auxin–JA molecular cross-talk in plant defense systems. ToLCNDV infection in tomato causes typical disease symptoms in the leaves such as curling, fusing of leaflets with no serrated edges similar to the phenotypes caused due to the overexpression of miR319 in *Arabidopsis* (Palatnik et al. 2003). Of interest, miR319 also controls JA pathway through the regulation of host transcripts TCP and LoxD (Schommer et al. 2008). To LCNDV infection induces the upregulation of miR319 along with the downregulation of target transcripts TCP1 and LoxD, suggesting viral infection entails auxin and JA signaling molecular cross-talk. It is further corroborated by the strong interaction of ToLCNDV-AC4 and host-derived SAG protein—a vital protein component of JA signaling. Notwithstanding the limitations of this study in corroborating the protein–protein interactions through bimolecular fluorescence complementation (BiFC) or co-immuno precipitation techniques, data obtained from Y2H assay, transcript(s) expression and phenotypic analysis substantially prove the cross-talk between ToLCNDV-AC4 and host auxin regulatory components. Thus, we conclude that strong interplay of auxin and JA biosynthesis pathways and their tight network underlies the synergistic effect on the plant during virus infection (Fig. 7).

Author contribution statement SP conceived and designed the experiments. VT, VS, NB, GK, VP, and AW performed the experiments. SP, VT, and SVR analyzed the data and wrote the paper. All authors have read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors wish to state that no conflicts of interest exist.

References

Bazzini AA, Hopp HE, Beachy RN, Asurmendi S (2007) Infection and co-accumulation of tobacco mosaic virus proteins alter microRNA levels, correlating with symptom and plant development. Proc Natl Acad Sci USA 104:12157–12162

Benjamins R, Scheres B (2008) Auxin: the looping star in plant development. Annu Rev Plant Biol 59:443–465

Carluccio AV, Prigigallo MI, Rosas-Diaz T, Lozano-Duran R, Stavolone L (2018) S-acylation mediates Mung bean yellow mosaic virus AC4 localization to the plasma membrane and in turns gene silencing suppression. PLoS Pathog 14:1007207

Chandler JW (2016) Auxin response factors. Plant Cell Environ 39:1014–1028

Chen K, Khatabi B, Fondong VN (2019) The AC4 protein of a cassava geminivirus is required for virus infection. Mol Plant Microbe Interact 32(7):865–875. https://doi.org/10.1094/mpmi-12-18-0354-r

Cheng Y, Dai X, Zhao Y (2006) Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissues in *Arabidopsis*. Genes Dev 20:1790–1799

Floková K, Tarkowská D, Miersch O, Strnad M, Wasternack C, Novák O (2014) UHPLC-MS/MS based target profiling of stress-induced phytohormones. Phytochemistry 105:147–157

Fondong VN (2019) The ever-expanding role of C4/AC4 in geminivirus infection: Punching above its weight? Mol Plant 12:145–147

Hipp K, Rau P, Schäfer B, Pfannstiel J, Jeske H (2016) Translation, modification and cellular distribution of two AC4 variants of African cassava mosaic virus in yeast and their pathogenic potential in plants. Virology 498:136–148

Islam W, Qasim M, Noman A, Adnan M, Tayyab M, Farooq TH, Wei H, Wang L (2018) Plant microRNAs: front line players against invading pathogens. Microb Pathog Internet 118:9–17

Jin L, Qin Q, Wang Y, Pu Y, Liu L, Wen X, Ji S, Wu J, Wei C, Ding B, Li Y (2016) Rice dwarf virus P2 protein hijacks auxin signaling by directly targeting the rice OsIAA10 protein, enhancing viral infection and disease development. PLoS Pathog 12(9):e1005847

Jyothsna P, Haq QMI, Singh P, Sumiya KV, Praveen S, Rawat R, Briddon RW, Malathi VG (2013) Infection of tomato leaf curl New Delhi virus (ToLCNDV), a bipartite begomovirus with betasatellites, results in enhanced level of helper virus components and antagonistic interaction between DNA B and betasatellites. Appl Microbiol Biotechnol 97:5457–5471

Kasschau KD, Xie Z, Allen E, Llave C, Chapman EJ, Krizan KA, Carrington JC (2003) P1/HC-Pro, a viral suppressor of RNA silencing, interferes with *Arabidopsis* development and miRNA function. Dev Cell 4:205–217

Koyama T, Mitsuda N, Seki M, Shinozaki K, Ohme-Takagi M (2010) TCP transcription factors regulate the activities of ASYMMETRIC LEAVES1 and miR164, as well as the auxin response, during differentiation of leaves in *Arabidopsis*. Plant Cell 22:3574–3588

Lang QL, Zhou XC, Zhang XL, Drabek R, Zuo ZX, Ren YL et al (2011) Microarray-based identification of tomato microRNAs and time course analysis of their response to Cucumber mosaic virus infection. J Zhejiang Univ Sci B 12(2):116–125

Li SB, Xie ZZ, Hu CG, Zhang JZ (2016) A review of auxin response factors (ARFs) in plants. Front Plant Sci 7:47

Li H, Zeng R, Chen Z, Liu X, Cao Z, Xie Q, Yang C, Lai J (2019) S-acylation of a geminivirus C4 protein is essential for regulating the CLAVATA pathway in symptom determination. J Exp Bot 69:4459–4468

Lopez JA, Sun Y, Blair PB, Mukhtar MS (2015) TCP three-way handshake: linking developmental processes with plant immunity. Trends in Plant Sci 20:238–245

Ludwig-Muller J (2011) Auxin conjugates: their role for plant development and in the evolution of land plants. J Exp Bot 62:1757–1773

Martín-Trillo M, Cubas P (2010) TCP genes: a family snapshot ten years later. Trends Plant Sci 15:31–39

Mashiguchi K, Tanaka K, Sakai T, Sugawara S, Kawaide H, Natsume M, Hanada A, Yaeno T, Shirasu K, Yao H, McSteen P (2011) The main auxin biosynthesis pathway in *Arabidopsis*. Proc Natl Acad Sci USA 108:18512–18517

Mei Y, Wang Y, Hu T, Yang X, Lozano-Duran R, Sunter G, Zhou X (2018a) Nucleocytoplasmic shuttling of geminivirus C4 protein mediated by phosphorylation and myristoylation is critical for viral pathogenicity. Mol Plant 11:1466–1481

Mei Y, Yang X, Huang C, Zhang X, Zhou X (2018b) Tomato leaf curl Yunnan virus-encoded C4 induces cell division through enhancing stability of Cyclin D 1.1 via impairing NbSKη-mediated phosphorylation in Nicotiana benthamiana. PLoS Pathog 14(1):e1006789. https://doi.org/10.1371/journal.ppat.1006789

Mills-Lujan K, Deom CM (2010) Geminivirus C4 protein alters *Arabidopsis* development. Protoplasma 239:95–110

Padmanabhan MS, Kramer SR, Wang X, Culver JN (2008) Tobacco mosaic virus replicase-auxin/indole acetic acid protein interactions: reprogramming the auxin response pathway to enhance virus infection. J Virol 82(5):2477–2485

Palatnik JF, Allen E, Wu X, Schommer C, Schwab R, Carrington JC, Weigel D (2003) Control of leaf morphogenesis by microRNAs. Nature 425:257–263

Paque S, Weijers D (2016) Q&A: Auxin: the plant molecule that influences almost anything. BMC Biol 14:67

Piroux N, Saunders K, Page A, Stanley J (2007) Geminivirus pathogenicity protein c4 interacts with *Arabidopsis thaliana* shaggy-related protein kinase AtSKeta, a component of the brassinosteroid signaling pathway. Virol 362:428–440

Praveen S, Ramesh SV, Mishra AK, Koundal V, Palukaitis P (2010) Silencing potential of viral derived RNAi constructs in Tomato leaf curl virus-AC4 gene suppression in tomato. Transgenic Res 19:45–55

Qi L, Yan J, Li Y, Jiang H, Sun J, Chen Q, Li H, Chu J, Yan C, Sun X, Yu Y (2012) *Arabidopsis thaliana* plants differentially modulate auxin biosynthesis and transport during defense responses to the necrotrophic pathogen *Alternaria brassicicola*. New Phytol 195:872–882

Ramesh SV, Mishra AK, Praveen S (2007) Hairpin RNA-mediated strategies for silencing of tomato leaf curl virus AC1 and AC4 genes for effective resistance in plants. Oligonucleotides 17:251–257

Rosas-Diaz T, Zhang D, Fan P, Wang L, Ding X, Jiang Y, Jimenez-Gongora T, Medina-Puche L, Zhao X, Feng Z, Zhang G (2018) A virus-targeted plant receptor-like kinase promotes cell-to-cell spread of RNAi. Proc Natl Acad Sci USA 115:1388–1393

Schommer C, Palatnik JF, Aggarwal P, Chételat A, Cubas P, Farmer EE, Nath U, Weigel D (2008) Control of jasmonate biosynthesis and senescence by miR319 targets. PLoS Biol 6:e230

Stepanova AN, Robertson-Hoyt J, Yun J, Benavente LM, Xie DY, Doležal K, Schlereth A, Jürgens G, Alonso JM (2008) TAA1-mediated auxin biosynthesis is essential for hormone crosstalk and plant development. Cell 133:177–191

Tagami Y, Inaba N, Kutsuna N, Kurihara Y, Watanabe Y (2007) Specific enrichment of miRNAs in *Arabidopsis thaliana* infected with Tobacco mosaic virus. DNA Res 14:227–233

Teng K, Chen H, Lai J, Zhang Z, Fang Y, Xia R, Zhou X, Guo H, Xie Q (2019) Involvement of C4 protein of Beet severe curly top virus (Family Geminiviridae) in virus movement. PLoS ONE 5(6):e11280

Vinutha T, Kumar G, Garg V, Canto T, Palukaitis P, Ramesh SV, Praveen S (2018) Tomato geminivirus encoded RNAi suppressor protein, AC4 interacts with host AGO4 and precludes viral DNA methylation. Gene 678:184–195

Weijers D, Wagner D (2016) Transcriptional responses to the auxin hormone. Annu Rev Plant Biol 67:539–574

Wieczorek P, Wrzesińska B, Obrępalska-Stęplowska A (2013) Assessment of reference gene stability influenced by extremely divergent disease symptoms in *Solanum lycopersicum* L. J Virol Methods 194:161–168

Wu L, Luo P, Di DW, Wang L, Wang M, Lu CK, Wei SD, Zhang L, Zhang TZ, Amakorová P, Strnad M (2015) Forward genetic screen for auxin-deficient mutants by cytokinin. Sci Rep 5:11923. https://doi.org/10.1038/srep11923

Zeng R, Liu X, Yang C, Lai J (2018) Geminivirus C4: interplaying with receptor-like kinases. Trends Plant Sci 23:1044–1046

Zhang Y, Friml J (2019) Auxin guides roots to avoid obstacles during gravitropic growth. New Phytol 225(3):1049. https://doi.org/10.1111/nph.16203

Zhao Y (2014) Auxin biosynthesis. Arabidopsis Book 12:e0173. https://doi.org/10.1199/tab.0173

Zhao Y, Hull AK, Gupta NR, Goss KA, Alonso J, Ecker JR, Normanly J, Chory J, Celenza J (2002) Trp-dependent auxin biosynthesis in *Arabidopsis*: involvement of cytochrome P450s CYP79B2 and CYP79B3. Genes Dev 16:3100–3112

Zhu S, Gao F, Cao X, Chen M, Ye G, Wei C, Li Y (2005) The rice dwarf virus P2 protein interacts with ent-kaurene oxidases in vivo, leading to reduced biosynthesis of gibberellins and rice dwarf symptoms. Plant Physiol 139(4):1935–1945