Identification of host cellular targets of AC4 and AV2 proteins of tomato leaf curl palampur virus and their sub-cellular localization studies

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Abstract Tomato leaf curl palampur virus (ToLCPaV) is a bipartite begomovirus with genome organization typical of old world begomoviruses. It infects commercially important crops and weeds in the Asian subcontinent. Apart from other proteins, the DNA-A of the virus encodes AV2 and AC4 proteins of approximately 13.73 and 6.7 kDa, respectively. In case of other begomoviruses, previous studies have shown the role of AV2 and AC4 proteins in virus movement, pathogenesis and suppression of gene silencing. However, the ToLCPaV proteins are significantly variable in comparison to closest relative and hence there is a need to work out their functions. In this study, we identified 9 cellular proteins of tomato that interact with AV2 and AC4 proteins, through yeast two hybrid screening. Upon sequence analysis, these interactors were identified as cysteine protease, katanin p60 ATPase-containing subunit A-like, guanine deaminase, NADH dehydrogenase (ubiquinone) iron-sulfur protein, glyceraldehyde-3-phosphate dehydrogenase B, 60S acidic ribosomal P0 protein, acyl co-A dehydrogenase IBR3, oxygen-evolving enhancer protein 1 and peroxisomal membrane protein 11D. These proteins play a vital role in protein degradation, plant defense response, microtubule severing,

photosynthesis and protein synthesis. The two viral proteins, however, did not interact with each other in yeast. AV2 when fused with GFP under the control of cauliflower mosaic virus 35S promoter was localized in nucleus and cytoplasm. On the other hand, AC4-GFP fusion was localized only in cytoplasm. The outcome of present study will help to elucidate the mechanism of viral pathogenesis. Further functional characterization of identified host proteins will provide an insight into their involvement in disease development.

Keywords Tomato leaf curl palampur virus · Protein–protein interaction · Yeast two hybrid analysis · Plant defense

Introduction

The genus *Begomovirus* belongs to *Geminiviridae* family and consists of viruses with ssDNA as genetic material encapsulated in geminate particles [8]. Geminivirus genome has bi-directional open reading frames (ORFs) that encode multifunctional proteins [17]. Due to a small number of encoded proteins, they rely on the host cell machinery to make suitable environment for their proliferation [7]. Currently, this family has been divided into nine genera; based on genome organization, transmission by insect vector and host range [52]. The genus *Begomovirus* has the largest number of species in the family, and the members are transmitted by a whitefly vector, *Bemisia tabaci*. Based on the number of genomic components present, these viruses are classified either as monopartite or bipartite. Bipartite begomoviruses have two genomic components designated as DNA-A and DNA-B,

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whereas monopartite begomoviruses have genome similar to DNA-A of bipartite begomoviruses [8].

Tomato leaf curl palampur virus (ToLCPaV), a bipartite begomovirus, was first reported from northern India, infecting tomatoes. The virus infection causes leaf curling, short internodes, reduced growth and yield [25]. Later, the virus was also reported from cucurbits, beans and weeds from India, Pakistan and Iran [2, 19, 31]. ToLCPaV DNA-A encodes six proteins; AV1-coat protein and AV2—pre-coat protein in sense orientation; AC1—replication initiator protein, AC2—transcription activator protein, AC3—replication enhancer protein and AC4 protein in the complementary sense orientation. DNA-B codes for BV1-nuclear shuttle protein in the sense, and BC1-movement protein, in the complementary sense orientation. The AV2 ORF partially overlaps with coat protein (CP) whereas the AC4 ORF is completely nested in the ORF of replication initiator protein.

In monopartite begomoviruses, the homologous V2 protein performs the function of virus movement but in bipartite begomoviruses BC1 act as movement protein. Further, V2 protein has been reported as RNA silencing suppressor and pathogenicity determinant in case of monopartite begomoviruses [27, 40, 53]. The AC4 is the least conserved protein among begomoviruses and has also been recognized as an RNA silencing suppressor in some isolates. AC4 has also been shown to play a significant role in infectivity and suppress the systemic phase of RNA silencing [12, 45, 49]. However, in other cases, the AC4 protein was found to have negligible role in virus infectivity [20, 38, 44]. Additionally, the role of AV2 and AC4 in ToLCPaV pathogenicity is still unknown and hence was investigated.

Yeast two hybrid (Y2H) is a powerful and reliable method developed to study protein–protein interactions [10]. In a LexA based yeast two hybrid system, the DNA binding domain (BD) is derived from LexA protein of *Escherichia coli* while the activation domain (AD) is obtained from acid blob domain B42. Separately, the LexA (BD) or B42 (AD) is unable to transcribe the reporter gene on nutritional selection; therefore the bait is fused with LexA, and prey or interactor library is fused with the B42 domain. An interaction between bait and prey protein fusion activates the reporter gene expression [15]. Y2H has been successfully utilized to dissect the virus–host interactions for understanding the molecular mechanism of virus infection. A screen for host proteins interacting with V2 protein of tomato yellow leaf curl virus (TYLCV) was based on Y2H, identified SGS3 as an interactor protein, and this interaction was required for the suppression of RNA silencing [14]. Y2H screening of arabidopsis cDNA library against C4 of beet curly top virus (BCTV), led to the identification of shaggy related protein kinase (AtSK η

and AtSK ζ) as potential interactors, involved in brassinosteroid signalling pathway [34].

To investigate the functions of AV2 and AC4 proteins in ToLCPaV pathogenicity, we performed Y2H screening of tomato cDNA library and identified the putative interacting host proteins. Furthermore, the sub-cellular localization of viral proteins was also studied. The correlation between sub-cellular localization and interactions of viral proteins with plant proteins is also discussed.

Materials and methods

Plant material and virus source

The genome of ToLCPaV (DNA-A Accession Number: AM884015) cloned in pBluescript KS (+) vector was used in the present study [25]. *Nicotiana benthamiana* was used as a model plant and grown in an insect-free growth chamber at 23 °C and 70% relative humidity under 16 h light and 8 h darkness regime.

Cloning of viral genes in yeast vectors

For the amplification of AV2 and AC4 ORFs, the cloned DNA-A of the virus was used as a template. In our previous work, full length AV2 protein was shown to autoactivate the reporter gene when fused with LexA binding domain, therefore we chose deletion mutant of AV2 in the present study. Primers were designed for the amplification of AC4, AV2 and a deletion mutant of AV2, lacking 10 amino acids from N-terminal to produce BD and AD fusion proteins (Table 1). These ORFs were amplified using cloned DNA-A as a template in a PCR reaction. The amplicons were cloned into pGEMT-easy vector and recombinant plasmids were double-digested with *EcoRI* and *XhoI* restriction enzymes to release the respective inserts. The inserts (AV2, AV2 Δ 10N, and AC4) were separately sub-cloned into yeast bait and prey vectors; pEG202 and pJG4-5, to make LexA and B42 fusion gene constructs. The recombinant plasmids; LexA-AV2 Δ 10N, LexA-AC4, B42-AV2, and B42-AC4 were sequenced for confirmation of correct orientation and integrity.

Yeast strain and control plasmids

Yeast strain EGY48 (MAT α trp1 his3 ura3 leu2::6 LexAop-LEU2) was used in this study and grown on yeast nitrogen base glucose (Clontech, TakaraBio, Japan) lacking uracil (YNB (glu)-ura) medium at 30 °C. EGY48 strain consists of two reporter markers; LEU2 (integrated in yeast chromosome) and LacZ (encoded by pSH18-34 plasmid). The interaction was analyzed by expression of reporter

Table 1 List of primers used in the present study

Primer	Gene/Vector	Primer Sequence 5'-3'	Study
P1	AC4	FP: <u>GAATTC</u> ATGGGTCTCCGCATATCCATGTC	Y2H
P2		RP: <u>CTCGAG</u> CTAAAACGTCTCCGTCTTTGTTCGATGT	
P3	AV2	FP: <u>GAATTC</u> ATGTGGGATCCATTATTGCACGAAT	Y2H
P4		RP: <u>CTCGAG</u> TCACAGCCCCCTGGGCACGTC	
P5	AV2Δ10N	FP: TGGAAATTCATGGAAAGCGTTCATGGTCTTAGGTG	Y2H
P6		RP: <u>CTCGAG</u> TCACAGCCCCCTGGGCACGTC	
P7	pEG202	FP: CGTCAGCAGAGCTTCACC	Sequencing of bait
P8		RP: GAAAGCAACCTGACCTACAG	
P9	pJG4-5	FP: CTGAGTGGAGATGCCTCC	Sequencing of prey
P10		RP: GCCGACAACCTTGATTG	
P11	AC4	FP: AACCATGGGTCTCCGCATATCCATGTC	Sub-cellular localization
P12		RP: AA <u>ACTAGT</u> AAACGTCTCCGTCTTTGTTCG	
P13	AV2	FP: CCATGGATATGTGGGATCCATTATTGCACGAAT	Sub-cellular localization
P14		RP: <u>CTCGAG</u> CAGCCCCCTGGGCACGTC	
P15	pCAMBIA1302	FP: GTCTCTTACGACTCAATGACAAG	Sequencing of GFP fusion
P16		RP: GGTAAGTTTTCCTGATGTTGC	

gene either on nutritional selective medium (leucine) or non-selective βX-gal supplemented medium. A LexA-bi-coid homeodomain fusion (pRHF1) is unable to activate the transcription, used as negative control, while pSH17-4 expressing LexA-GAL4 activation domain is used as positive control in the present study.

Yeast two hybrid library screening

Tomato cDNA library cloned in the pJG4-5 vector was provided as a kind gift from Prof. Yedidya Gafni, ARO Volcani centre, Israel. LexA-AV2Δ10N and LexA-AC4 plasmids were separately co-transformed with cDNA library using lithium acetate/Polyethylene glycol (LiOAc/PEG) method. Yeast transformants were selected on yeast nitrogen base galactose lacking uracil, histidine, tryptophan and leucine (YNB (gal)-ura, -his, -trp, -leu) medium and incubated at 30 °C for 5–10 days until the appearance of colonies. Selection of interactors was done by serial dilutions of the co-transformed yeast on YNB (gal)-ura, -his, -trp, -leu plates. Colonies found positive in dilution assay were inoculated in a YNB(glu)—ura, -trp, -his medium for total plasmid extraction. The total plasmid content was retransformed in KC8 (pyrF, leuB600, trpC, hisB463) strain of *E. coli* and individual library plasmids containing cDNA fragment of tomato were rescued. The library plasmid was identified by digesting with *Eco*RI and *Xho*I restriction enzymes. Further, the specificity of interaction was confirmed by one to one interaction of the library plasmid with LexA-AV2Δ10N and LexA-AC4 plasmids by dilution assay on YNB (gal)-ura, -his, -trp, -leu plates. The

potential positive AV2 and AC4 interactors were sequenced using pJG4-5 specific primers (Table 1).

Filter assay for β-galactosidase

Yeast colonies were patched on YNB (gal)-ura, -trp, -his plates and incubated at 30 °C for 2 days. A fresh Whatman 3 MM filter paper was placed on the yeast colonies and gently removed. The filter was placed in liquid N₂ for 5 min and kept at 37 °C for 5 min. This step was repeated thrice for lysis of yeast cells. A filter paper was wetted with Z buffer (60 mM Sodium phosphate (dibasic), 40 mM Sodium phosphate (monobasic), 10 mM Potassium chloride, 1 mM Magnesium sulphate) supplemented with X-gal (0.01 mg/ml in DMSO) and the filter containing lysed yeast cells was placed on pre-wet filter for 2–3 h at 30 °C in dark. The colonies were observed for the appearance of blue colour.

Sub-cellular localization of viral proteins

To determine the sub-cellular localization of AV2 and AC4 proteins, the viral genes were amplified using the primers described in Table 1. The amplicons were cloned into pGEMT easy vector and further sub-cloned in a binary vector, pCAMBIA1302 under the control of a 35S promoter, in fusion with GFP at C-terminal of AV2 and AC4 proteins. These constructs were designated as 35S::AV2-GFP and 35S::AC4-GFP and pCAMBIA1302 vector (35S::GFP) was used as negative control. All constructs were separately transformed in *Agrobacterium* strain

GV3101, and positive colonies were screened by colony PCR. The *Agrobacterium* colonies were grown overnight in LB media supplemented with kanamycin (50 µg/ml) and rifampicin (25 µg/ml), the same was pelleted down by centrifugation and re-suspended in infiltration medium (10 mM MgCl₂, 10 mM MES pH 5.6 and 150 µM acetosyringone). The final OD 600 of the culture was set at 0.5 and onion epidermal peel was transformed with *Agrobacterium* cultures [43]. The scales were briefly rinsed with distilled water and observed under the confocal microscope (CarlZeiss LSM510, Germany). For sub-cellular localization in *N. benthamiana*, the *Agrobacterium* cells suspended in infiltration medium were kept at room temperature for three hours and pressure infiltrated in *N. benthamiana* leaves using 1 ml syringe. After 72 h post inoculation, leaves were observed for GFP fluorescence under the confocal microscope. The fluorescence of GFP was observed at 488/510 nm excitation/emission wavelengths.

Results

AV2 does not associate with AC4 in yeast

Prior to library screening, autoactivation potential of the bait proteins (AV2 and AC4), was examined using the expression of the reporter gene in auxotrophic yeast EGY48. The results showed that AV2 could autoactivate the expression of a reporter gene. Therefore, a deletion mutant of AV2 lacking the autoactivation domain was used for further studies (unpublished results). However, the AC4 protein did not show transactivation in yeast dilution assay on YNB (gal)-ura, -his, -leu medium. Furthermore, the yeast colonies co-transformed with LexA-AC4 and B42-AV2 recombinant plasmids were unable to grow on YNB(gal)-ura, -his, -trp, -leu medium, up to 10⁻⁴ dilution. The yeast cells transformed with pSH17-4 + pJG4-5 (positive control) showed growth, whereas negative controls (LexA-AC4 + pJG4-5 and pRHFM1 + B42-AC4) were unable to grow on nutritional drop-out medium (Fig. 1). These results indicate absence of interaction between AV2 and AC4 proteins in yeast cells.

Identification of interactors through Y2H

A tomato cDNA library, fused to B42 acid blob domain, was screened using LexA-AV2Δ10N and LexA-AC4 as a bait in Y2H assay. A total 4 × 10⁶ transformants were obtained and 30 colonies were selected as candidate clones on YNB(gal)-ura, -his, -trp, -leu medium. The yeast plasmid extracted from 30 colonies were transformed into KC8 strain of *E. coli* and the individual library plasmids were re-transformed in yeast EGY48 along with LexA-AV2Δ10N

Fig. 1 Interaction between LexA-AC4 and B42-AV2 in a Yeast two-hybrid system. The yeast strain EGY48 possessing indicated constructs were spotted on YNB (gal)-ura, -trp, -his, -leu medium with 10-fold serial dilutions and β-galactosidase filter assay was performed on YNB (gal)-ura, -trp, -his medium supplemented with βX-gal. pRHFM1 + B42-AC4 and pSH17-4 + pJG4-5 were used as negative and positive controls, respectively

or LexA-AC4 constructs. Out of 30 clones, only 12 clones showed interaction after re-transformation of prey plasmids in the yeast on YNB (gal)-ura, -his, -trp, -leu medium and β-galactosidase assay (Fig. 2). The cDNA clones were sequenced, and the fusion of B42 domain with cDNA clones was analyzed. The interactors were identified by their similarity to available sequences in BLAST search at NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The yeast two hybrid screening resulted in the identification of nine putative interactors; cysteine protease, katanin p60 ATPase-containing subunit A-like, guanine deaminase, NADH dehydrogenase (ubiquinone) iron-sulfur protein, glyceraldehyde-3-phosphate dehydrogenase B, 60S acidic ribosomal protein P0 protein, acyl co-A dehydrogenase IBR3, oxygen-evolving enhancer protein 1 and peroxisomal membrane protein 11D. The details of identified prey proteins showing interaction with AV2 and AC4 proteins in Y2H screening are listed in Table 2.

AV2 interacts with host proteins involved in defense, metabolism and protein synthesis

Two cDNA clones were found similar to *S. lycopersicum* cysteine protease TDI-65 (accession NM_001247395) in BLAST search, and 273 amino acids of the c-terminal region were involved in interaction with the AV2Δ10N protein. Partial c-terminal cDNA clones of *S. lycopersicum* Katanin p60 ATPase-containing subunit A-like 2 (accession XM_004233080) and 60S ribosomal protein P0 like (accession XM_004239733) possessing 154 and 74 aa residues, respectively showed strong interaction with AV2 protein. Two cDNA clones were identified as *S. lycopersicum* glyceraldehyde-3-phosphate dehydrogenase B, chloroplastic (accession XM_004238398) and 368 aa residues of N-terminal region were involved in the interaction with the viral protein. Full length cDNA clones of *S. lycopersicum* NADH

Fig. 2 Yeast two hybrid interaction analysis for identification of interactors. The EGY48 strain was co-transformed with indicated bait and prey plasmids. The 10-fold dilution was performed on YNB (gal)-ura, -trp, -his, -leu medium and β -galactosidase filter assay was performed on YNB(gal)-ura, -trp, -his medium. pRFHM1 + pSH18-34 and pSH17-4 + pJG4-5 were used as negative and positive controls, respectively

dehydrogenase [ubiquinone] iron-sulfur protein 8, mitochondrial (accession XM_004230878) and *S. pennellii* guanine deaminase (accession XM_015232580) comprising of 229 and 186 aa, respectively were identified as interactors of AV2 through BLAST analysis.

AC4 interacts with host proteins involved in photosynthesis and cellular metabolism

In yeast two hybrid screening, three putative proteins of tomato were identified as interactors. Two cDNA clones of 1200 bp length, encoding 337 amino acids were identified as *S. lycopersicum* oxygen-evolving enhancer protein 1 (OEE1); (accession NP_001296294) in NCBI Blast search. The sequence analysis revealed that 337 amino acids represent full length OEE1 protein except for first methyl group. OEE1 is a 33 kDa thylakoid membrane protein and is involved in oxygen evolution during photosynthesis. It stabilizes manganese cluster in water splitting site [30]. Another

cDNA clone was 2083 bp long that codes for 663 amino acids and the sequence was found similar to *S. lycopersicum* putative acyl-CoA dehydrogenase IBR3 (accession XP_004239801). It shows 71% sequence identity with *Arabidopsis thaliana* acyl-CoA dehydrogenase-like protein (accession NP_187337). IBR3 may be involved in the peroxisomal beta-oxidation of Indole-3 butyric acid (IBA) to Indole 3 acetic acid (IAA) and it also possesses peroxisomal localization sequence at the c-terminal [54]. The third interactor of AC4 was identified as a peroxisomal protein that showed 97% sequence similarity to c-terminal 63 amino acids of peroxisomal membrane protein 11D (PEX 11) from *S. lycopersicum* (accession XP_004229845). PEX 11D plays a pivotal role in peroxisomal biogenesis [32].

Localization of AV2 and AC4 proteins

Confocal analysis showed that AV2-GFP fusion was localized in both cytoplasm and plasma membrane of the

Table 2 List of *S. lycopersicum* proteins interacting with AV2 and AC4 proteins in Y2H assay

Protein description and pathway	CDNA Interactor size (bp)	Identity (%)	Accession number
Protein degradation and plant defense			
<i>Solanum lycopersicum</i> cysteine protease TDI-65 (tdi-65) ^a	~ 1000	100	NM_001247395
Microtubule-severing enzyme			
<i>S. lycopersicum</i> katanin p60 ATPase-containing subunit A-like 2 (LOC101249324) ^a	~ 800	99	XM_004233080
Protein synthesis			
<i>S. lycopersicum</i> 60S acidic ribosomal protein P0 (LOC101261521) ^a	~ 400	99	XM_004239733
Cellular metabolism			
<i>S. lycopersicum</i> glyceraldehyde-3-phosphate dehydrogenase B, chloroplastic (LOC101264004) ^a	~ 1500	97	XM_004238398
<i>S. lycopersicum</i> NADH dehydrogenase [ubiquinone] iron-sulfur protein 8, mitochondrial (LOC101257611) ^a	~ 1000	99	XM_004230878
<i>S. pennellii</i> guanine deaminase (LOC107031269) ^a	~ 800	98	XM_015232580
<i>S. lycopersicum</i> acyl-CoA dehydrogenase IBR3 ^b	~ 2100	98	XP_004239801
<i>S. lycopersicum</i> peroxisomal Membrane Protein ^b	~ 200	97	XP_004229845
Photosynthesis			
<i>S. lycopersicum</i> Oxygen-evolving enhancer protein 1 (OEE1) ^b	~ 1200	99	NP_001296294

The BLAST search was performed for the putative proteins at NCBI database

^aand ^b represents the interacting proteins of AV2 and AC4, respectively

onion epidermal cells, whereas free GFP was distributed throughout the cell (Fig. 3). The AC4-GFP fusion was localized as punctate bodies in the cytoplasm of *N. benthamiana* epidermal cells (Fig. 4). These results showed different compartmentalization of AV2 and AC4 proteins in the host cell, possibly indicating their varied role during virus infection.

Discussion

Viruses are completely dependent on the host cell machinery for their survival. There is a continuous arms-race between the viruses and host for survival. Viral proteins interact with various host factors and favour the replication and multiplication of the virus. Hence, identification of host factors which interact with the viral proteins provide clue about the complexity of host defense mechanisms and viral pathogenesis. Moreover, host-pathogen interaction studies reveal the molecular basis of virus infection that will be helpful towards the development of virus resistance strategies. Upon sequence analysis of the identified prey, we found that these proteins are involved in protein degradation, defense, microtubule severing, cellular metabolism, protein synthesis and photosynthesis. However, both viral proteins do not show interaction with each other in yeast, indicating their independent role in contribution towards viral virulence.

Cysteine protease (CYP1) is classified as papain like cysteine protease, involved in the protein degradation, programmed cell death (PCD), senescence and immunity in plants [26, 41]. CYP1 possesses 3 domains; cysteine protease, proline rich and granulin domain, respectively [26]. The present study showed that AV2 interacts with C-terminal domains; cysteine protease and granulin of CYP1 and similar findings were obtained during the interaction between V2 of monopartite begomovirus (TYLCV) and CYP1 [5, 6]. Previously, the cysteine protease TDI-65 was found to localize in nuclei and chloroplast under drought stress [46]. Our findings suggest that the presence of AV2 in the nucleus of the host cell and its interaction with CYP1 might be involved in the transcriptional regulation of host genes involved in defense pathways. Katanin p60 ATPase-containing subunit A-like protein (Kat) performs the severing of microtubules in an ATPase dependent manner to control the cell division in plants [35]. Kat has two subunits; larger (p80) and smaller (p60); the p80 directs the p60 subunit to intracellular sites while the p60 subunit possesses an enzymatic ATPase activity [18]. Association of the Katanin (154 aa) with AV2 suggests its probable role in regulating host cell division to promote virus movement. Previous findings have shown the inhibition of Katanin activity by plant protein TORTIFOLIA1 (TOR1) that favours the spread of tobacco mosaic virus (TMV) in *Arabidopsis* [33]. The viral protein interacts with a 60S acidic ribosomal protein P0 is (RPP0) that is a component of

Fig. 3 Sub-cellular localization of AV2 protein. Onion epidermal peels were transformed with 35S::GFP and 35S::AV2-GFP plasmids and expression of GFP was analyzed under the confocal microscope. Arrows depict the localization of AV2-GFP fusion in the cytoplasm and nucleus and a, b, c images corresponds to GFP, merged and bright field. Scale bar: 20,000 nm

ribosomal stalk [4]. In a previous study, the RPP0 protein has been shown to promote translation of viral proteins and enhance the potato virus A (PVA) infection in the host [16]. Furthermore, infection of *Plasmodiophora brassicae* in *Arabidopsis* resulted in down-regulation of RPP0 protein which suggested that RPP0 plays a crucial role in biotic stress [9]. Based on these findings we speculate that the AV2 might be involved in the regulation of translation process. Glyceraldehyde-3-phosphate dehydrogenase B (GAPDH) is a key enzyme involved in the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate [36]. GAPDH consists of three subunits; GAPDH-A and

GAPDH-B, found in the chloroplasts and GAPDH-C found in the cytoplasm [11]. In the present study, the N-terminal of GAPDH-B subunit was found to interact with AV2 protein in Y2H screening. The previous studies have shown the role of GAPDH in the export of RNA, replication and DNA repair [13] and microtubule assembly [50]. Furthermore, GAPDH-A was shown to facilitate intracellular movement of red clover necrotic mosaic virus (RCNMV) by interacting with virus movement protein in plant [22]. Similar results were also obtained in case of bamboo mosaic virus (BaMV) and tobacco bushy stunt virus (TBSV) infection, where the viral proteins bind with

Fig. 4 Sub-cellular localization of AC4 protein. *N. benthamiana* leaves were infiltrated with 35S::GFP and 35S::AC4-GFP constructs and expression of GFP was analyzed under the confocal microscope. Arrow depicts the localization of AC4-GFP fusion as punctate bodies in the cytoplasm and a, b, c images corresponds to GFP, merged and bright field. Scale bar: 20,000 nm

GAPDH to promote replication of viral RNA [37, 51]. NADH dehydrogenase [ubiquinone] iron-sulfur protein constituted the core subunit of the mitochondrial membrane respiratory chain complex I and involved in the transfer of electrons from NADH to the respiratory chain. The current study has shown the interaction of full length NADH dehydrogenase [ubiquinone] iron-sulfur protein with AV2 protein in yeast. Similar interaction results were obtained in case of P10 protein of southern rice black-streaked dwarf virus (SRBSDV) and NADH dehydrogenase subunit1 in Y2H assay [48]. Furthermore, the expression of NADH dehydrogenase transcripts was downregulated by a viral pathogenicity protein β C1 of chilli

leaf curl betasatellite (ChLCB) [3]. In addition, we reported the interaction between guanine deaminase and AV2 of ToLCPaIV. Guanine deaminase is involved in the deamination of guanosine and 2'-deoxyguanosine into xanthosine of purine alkaloid synthesis pathway [23]. However, the role of guanine deaminase during the biotic stress is yet to be deciphered.

An oxygen evolving complex (OEC), also known as water splitting complex, possesses three proteins of 33, 23 and 16 kDa, located in the lumen of chloroplast thylakoids. OEC is the site for water oxidation and evolution of molecular oxygen during light reaction of photosynthesis [30]. In this study, we have identified OEE1, a 33 kDa

protein, as an interactor of AC4 protein. OEE1 is a nuclear-encoded protein and is crucial for the stability of photosystem II (PS II) [28]. Besides the significant role in photosynthesis, the OEE1 protein from green algae, *Capsosiphon fulvescens* also possess anti-oxidant activity [24]. In agreement with our results, the previous study has demonstrated the interaction between RNA helicase domain of TMV replicase protein and 33 kDa protein of OEC in a yeast two hybrid assay. Further, silencing of this gene employing tobacco rattle virus based vector enhanced the TMV accumulation by 10-fold, and the virus infection resulted in downregulation of OEE1 transcript [1]. Additionally, alternanthera mosaic virus triple gene block 3 (TGB3) protein also showed strong interaction with OEE1 of the host in Y2H analysis and bimolecular fluorescence complementation (BiFC) assay. These findings suggest that OEE1 negatively regulates the virus infection and further studies are needed to functionally validate its role during ToLCPaV infection. In a yeast two hybrid assay, acyl-CoA dehydrogenase (IBR3) was found an AC4 interacting protein. Besides the possible involvement of peroxisomal beta-oxidation of Indole-3 butyric acid (IBA) to Indole 3 acetic acid (IAA); [42, 54], IBR3 was also shown to play a defense role in *A. thaliana* and up-regulated upon virulent *Pseudomonas syringae* pv. *Tomato*(Pst) DC3000 infection. IBR3 over-expressing lines of *Arabidopsis* displayed enhanced susceptibility to the bacteria while the mutant lines were resistant to pathogen infection [21]. Based on previous findings, we speculate that IBR3-AC4 interaction might be acting as a key player in the establishment of ToLCPaV infection.

In the present study, we demonstrated that AV2-GFP fusion localizes in the nucleus and cytoplasm of the onion epidermal cells. These results suggest that AV2 can shuttle between nucleus and the cytoplasm. In previous studies, the localization of monopartite V2 protein has been observed in cytoplasm, cell periphery, and the cytoplasmic aggregates [29, 39]. Based on these results, we suggest that AV2 recruits the host transcription machinery and also interacts with the cytoplasmic proteins of host. Confocal image analysis showed that AC4-GFP fusion localizes in the cytoplasm of *N. benthamiana* leaves. Contrastingly, the AC4/C4 protein of east African cassava mosaic Cameroon virus(EACMCV) and BCTV binds to the plasma membrane as well as to cytosolic membranes but is absent in nucleus. Further, it has been shown that N-myristoylation of the protein is found critical for membrane binding and pathogenicity [12, 34]. In Beet severe curly top virus (BSCTV) C4 protein was localized both in the cytoplasm and nucleus but is excluded from cell membranes [47]. These observations show that the localization of AC4/C4 protein among geminiviruses is not conserved that could be

due to different functions of this protein during viral infection.

To conclude, our study demonstrates the interaction of viral proteins with host proteins involved in various pathways of host and these interactions present an overview of the host defense system during ToLCPaV infection. Further studies are required to understand the role of interacting host proteins in plant-virus interactions and development of virus resistance.

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