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A thioredoxin-like protein of *Bemisia tabaci* interacts with coat protein of begomoviruses

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Abstract

Bemisia tabaci (whitefly) is the sole vector of begomoviruses, which transmits them in a persistent and circulative manner from infected to healthy plants. During this process, begomoviruses interact with various proteins in the insect vector *B. tabaci* that would play a specific role in the virus transmission. Identification and characterization of such proteins are important to understand the complete process of virus transmission. Coat protein (CP) of begomoviruses is the only protein which is reported to interact with proteins of the insect vector *B. tabaci*. In this study, we performed yeast two-hybrid assay using CP of cotton leaf curl Rajasthan virus (CLCuV) and *Tomato leaf curl New Delhi virus* (ToLCNDV) as bait in separate experiments and cDNA prepared from total RNA of *B. tabaci* was used as prey. Yeast two-hybrid assay resulted in identification of a thioredoxin-like protein (TLP) from CLCuV yeast two-hybrid library. Later TLP was also found to interact with CP of ToLCNDV. In vitro pull-down assay showed TLP interaction with CP of both CLCuV and ToLCNDV. TLP was found to interact with ToLCNDV virus particles isolated from tomato leaves.

Keywords Begomoviruses · *B. tabaci* · Thioredoxin-like protein · Yeast two-hybrid assay · In vitro pull-down assay · Dot blot assay

Introduction

Begomovirus is the largest genus among viruses and belongs to the family *Geminiviridae* [1, 2]. Geminiviruses are characterized by twinned icosahedral particles with circular ssDNA, which cause severe crop losses around the globe [3]. Geminiviruses are classified into nine genera, viz., *Begomovirus, Curtovirus, Mastrevirus, Topocuvirus, Becurtovirus, Turncurtovirus, Eragrovirus, Capulavirus*, and *Grablovirus*, on the basis of insect vector and genome organization [3–5]. Begomoviruses can have either monopartite genome (DNA-A) or bipartite genomes (DNA-A and DNA-B) [3]. *Cotton leaf curl virus, Tomato leaf curl virus* (ToLCV), and ToLCNDV are the major begomoviruses found in India. CLCuV causes cotton leaf curl disease (CLCuD) and ToLCNDV causes tomato leaf curl disease (TLCD) [6–8]. ToLCNDV has bipartite genome (both DNA-A and DNA-B components), whereas CLCuV has a monopartite genome [6, 8–11]. Monopartite begomoviruses are associated with alphasatellite DNA and/or betasatellite DNA. DNA-A contains six open reading frames (ORFs; AC1, AC2, AC3, AC4, AV1 and AV2). DNA-B contains two ORFs (BV1 and BC1) for symptom development and viral movement proteins in host plant [3, 12].

Bemisia tabaci is the sole vector of begomoviruses, which transmits them in a persistent and circulative manner from infected to healthy plants during their feeding behavior [13]. Upon feeding on infected plants, *B. tabaci* ingests begomoviral particles into the alimentary canal along with plant sap. Begomoviral particles subsequently reach the filter

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chamber and midgut region. Here begomoviruses cross the gut membrane and enter into hemolymph. Virion particles reach to salivary glands, penetrate it and settle in salivary duct. During subsequent feeding cycle, begomoviruses are transmitted to healthy plants, when they are egested out with saliva [14, 15]. There is very limited information available about specific interaction of begomoviral proteins and their interacting protein partners in *B. tabaci*. The coat protein (CP) of the virus is the only known factor required for viral transmission and changes in amino acid sequence of CP alter insect specificity and viral transmission by insect vector [16–18]. *B. tabaci* 16 kDa heat shock protein (BtHSP16) and 70 kDa heat shock protein were identified to interact with CP of Begomoviruses [19, 20]. A midgut protein (MGP) was shown to help in ToLCV transmission by the insect vector *B. tabaci* [21]. Recent studies report the important role of *knottin-1* gene and cyclophilin B protein in *Tomato yellow leaf curl virus* (TYLCV) transmission [22, 23]. *B. tabaci* peptidoglycan recognition protein (BtPGRP) has a potential binding site for TYLCV [24]. Another study suggested the role of clathrin-mediated endocytosis in TYLCV transmission [25]. GroEL proteins of endosymbiotic bacteria *Hamiltonella* and *Arsenophonus* were identified to interact with TYLCV and CLCuV, respectively, and *Hamiltonella* GroEL was shown to facilitate TYLCV transmission [7, 26]. Despite these results, the complete process of begomovirus transmission in molecular detail is still to be clearly understood.

In this study, we focused on identification of possible interacting protein partners for the CP of begomoviruses. For this, we performed yeast two-hybrid assays between cDNA prepared from total RNA isolated from whole body of *B. tabaci* as prey and CP of CLCuV and ToLCNDV as bait. Sequence analysis of positive clones resulted in their identification or similarity to various families of proteins. Later, complete ORF was generated by 5′ rapid amplification of cDNA end (5′ RACE) for one of the protein identified from CLCuV library, named thioredoxin-like protein (TLP). TLP was also found to interact with CP of ToLCNDV in yeast two-hybrid assay. Complete *tlp* gene was cloned into heterologous expression vector and the TLP was expressed and purified. In vitro pull-down experiments and dot blot assay were performed to validate interaction of TLP with CP of both CLCuV and ToLCNDV.

Materials and methods

Sample collection

Bemisia tabaci samples were collected from Insect Culture Facility at Indian Agricultural Research Institute (IARI), Pusa, New Delhi.

Yeast two-hybrid assay

Yeast two-hybrid assay was performed as mentioned by Rana et al. [21]. Briefly, yeast two-hybrid assay was performed by Matchmaker™ Library Construction and Screening Kit according to instructions provided by kit manufacturer with few modifications (Clontech Laboratories, Inc., USA). The cDNA was prepared from total RNA isolated from the whole *B. tabaci*. Genes of CP of CLCuV and ToLCNDV were cloned between *Bam*H1 and *Sal*1 restriction site of pGBKT7 vector for being used as baits. Yeast cells AH109 were co-transformed with *Sma1*-linearized pGADT7-Rec vector, cDNA library, and bait vectors (pGBKT7-CLCuV CP and pGBKT7-ToLCNDV CP in separate experiments). These cells were plated on two dropout (DO) media lacking leucine and tryptophan. These plates were incubated for 3–4 days at 30 °C to grow positive transformed cells. Most of the obtained colonies were again re-streaked on the 3DO (Leu^- Trp^- His^-) and 4DO (Leu^- Trp^- His^- Ade^-) media. Colonies, which were able to grow on the both 3DO and 4DO, were considered as positive clones. To re-confirm positive clones, β-gal assay was also performed. Plasmid DNAs from positive clones were isolated and *E. coli* DH5α cells were transformed with these plasmid DNAs. Selection markers were used for pGADT7-Rec vectors with gene of interest to get cDNA sequence. Plasmids were sent for commercial sequencing (Macrogen, South Korea) using specific primers. The sequences were submitted to BLASTn and BLASTx analysis in NCBI server [27–30]. AH109 cells were again co-transformed with selected identified positive clone and pGBKT7-ToLCNDV CP.

Generation of complete ORF of *tlp* by 5′ RACE

Total RNA was isolated from 30 *B. tabaci* using Ribozol™ RNA Extraction Reagent according to manufacturer's protocol (Amresco®, Solon, Ohio). To generate complete cDNA sequence of *tlp* gene, 5′ RACE was performed with two forward primers, Abridged Anchor Primer (AAP) and Abridged Universal Amplification Primer (AUAP) (Invitrogen, Carlsbad, USA), and gene-specific reverse primers, TLP-R1 and TLP-R2 (Table 1). Amplified 5′ RACE product was cloned into pGEM®-T Easy vector (Promega, Madison, WI, USA) and *E. coli* DH5α cells were transformed with these plasmids. Plasmid was isolated from three clones and sent for sequencing (Macrogen, South Korea). Yeast two-hybrid library sequence and 5′ RACE sequence were assembled together and were submitted to BLASTn analysis in NCBI server [27–30]. BLASTx and BLASTp analyses were done to find conserved region in

Table 1 Different primers used in this study

Primer names	Sequence (5′–3′)	T_m (°C)	References
AAP	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG	55	Invitrogen manual, Catalog no. 18374-058
AUAP	GGCCACGCGTCGACTAGTAC	55	Invitrogen manual, Catalog no. 18374-058
TLP-R1	TTACAAATCTGGGCGGACTTTGAAACC	53	This study
TLP-R2	TTCTGCTGCATACCTTCCACTTCT	53	This study
TLP-F	TCGAATTCCATATGCAATCGAGTCTTCGTAAGGTG	53	This study
TLP-Ra	GACGTCGACTTACAAATCTGGGCGACTTTGAAACC	53	This study
CLCuV CP F	CCGGATCCATGTCGAAGCGAGCTGC	55	[7]
CLCuV CP R	GACGTCGACTCAATTCGTTACAGAGTC	55	[7]
ToLCNDV CP F	AGATCTATGGCGAAGCGACCAGC	55	[34]
ToLCNDV CP R	GTCGACTTAATTTGTGACCGAATC	55	[34]

protein. ProtIdent and SignalP 4.1 of ExPASy portal were used for protease detection and signal peptide prediction [31, 32]. For the structure prediction of TLP, its amino acid sequence was subjected to RaptorX [33].

Cloning, expression, and purification of TLP

For cloning of full ORF of *tlp* gene, first total RNA was isolated from *B. tabaci* and cDNA was prepared. Then full ORF of *tlp* gene was amplified by PCR using TLP-F and TLP-Ra primers (Table 1). The amplified *tlp* gene was cloned into pGEM®-T Easy (Promega, Madison, WI, USA) by TA cloning according to manual instructions and *E. coli* DH5α transformed with these plasmid. Positive colonies were selected with blue/white colony screening. Plasmids were isolated from positive colonies using mdi pDNA Miniprep Kit according to instructions given in manual (Advanced Microdevices Pvt. Ltd.) and restriction digestion was performed with *Nde1* and *Sal1* restriction enzyme to further reconfirm positive clones. Plasmids from positive clones were sent for commercial sequencing (Macrogen, South Korea). The digested *tlp* gene product was purified from agarose gel using mdi Gel Extraction Kit according to instructions given in manual (Advanced Microdevices Pvt. Ltd.) and used for sub-cloning into pET-28a expression vector between *Nde1* and *Sal1* restriction sites. *E. coli* DH5α cells were transformed with cloned *tlp* gene in pET-28a vectors. Plasmids from transformed cells were isolated and were checked for the presence of *tlp* gene by restriction digestion with *Nde1* and *Sal1* restriction enzymes. Plasmids tested positive in restriction digestion were sequenced. Sequence analysis was performed to check proper gene frame and the absence of any mutations.

E. coli BL21 cells were transformed with pET-28a-*tlp* construct for heterologous expression. Few positive transformed colonies were grown at 37 °C individually to an OD_{600} (0.6) and IPTG was added to final concentration of 0.5 mM. Induced cultures were incubated at 180 rpm for 16 h at 18 °C for TLP expression. Inductions were checked by SDS-PAGE. Induced cells were centrifuged at 7000 rpm for 5 min at 4 °C to get pellet. The pellets were re-suspended into buffer A [20 mM Sodium phosphate (pH 7.5), 500 mM NaCl, 1% Triton, 1% NP40, 14 mM 2-mercaptoethanol (β-ME), 1 × protease inhibitor, and 5 mM Imidazole] and lysed by sonication at 30% amplitude. Lysate centrifuged at 7000 rpm for 20 min at 4 °C. Supernatant was incubated with pre-equilibrated Ni–NTA bead with buffer A without detergents (Triton and NP40) for 2 h at 4 °C on Nutator (ARON Laboratory Limited, Taiwan). Then beads were washed with washing buffer [20 mM sodium phosphate (pH 7.4), 500 mM NaCl, 1% Triton 100X (v/v), 1 × protease inhibitor, and 50 mM imidazole] twice. Bound protein fractions were eluted with elution buffer [20 mM sodium phosphate (pH 7.4), 500 mM NaCl, 1 × protease inhibitor, and 500 mM imidazole]. Eluted protein fractions were checked by SDS-PAGE and dialysis was performed with buffer B [50 mM Tris–Cl (pH 7.5), 300 mM NaCl, 20% glycerol, and 1 × protease inhibitor]. The protein fractions were stored at − 70 °C for later use.

Cloning, expression, and purification of CP of both CLCuV and ToLCNDV

The CPs of CLCuV and ToLCNDV were cloned, expressed, and purified as described previously for CLCuV CP [7, 34]. Briefly, *cp* genes of CLCuV and ToLCNDV were amplified with *cp* gene-specific primers

(Table 1) using CLCuV and ToLCNDV infected *B. tabaci* genomic DNA as template, respectively. Amplified PCR products were cloned into pGEM®-T Easy (Promega, Madison, WI, USA) and sent for commercial sequencing (Macrogen, South Korea) for confirmation. Further, both *cp* genes were sub-cloned into pGEX-4T1 expression vector (Amersham Biosciences, USA) and sequenced commercially (Macrogen, South Korea). *E. coli* strain [*Rosetta-gami 2*(*DE3*)*pLysS*] was transformed by expression vectors with *cp* gene insert, which subsequently were induced with 0.5 mM IPTG for 12 h at 18 °C. Induced cell pellets were dissolved in Buffer A [25 mM Tris–Cl (pH- 8.0), 75 mM NaCl, 2.5 mM EDTA, 5.0 mM $MgCl_2$, 2.5 mM DTT, 1 × protease inhibitor, 10% glycerol] and lysed by sonication at 30% amplitude. Soluble fractions of lysate were incubated with Glutathione Sepharose beads (GE Healthcare Life Sciences). Bound glutathione *S*-transferase (GST)-tagged CPs were eluted with elution buffer (Buffer A with 10 mM reduced glutathione) and subjected to dialysis with buffer B [25 mM Tris–Cl (pH- 8.0), 75 mM NaCl, 2.5 mM EDTA, 5.0 mM $MgCl_2$, 2.5 mM DTT, 25% glycerol]. Dialyzed CPs of both CLCuV and ToLCNDV were identified by western blotting using anti-ToLCNDV CP polyclonal antibody generated in rabbit and stored at − 70 °C for further use.

Generation and purification of anti-TLP antibody

For anti-TLP antibody generation, two 7-week-old BALB/c mice were used. Pre-immune blood was collected 1 week before injecting antibody. Blood was left for 1 h at room temperature and subsequently stored at 4 °C for overnight to separate blood cells with sera. Blood was subjected to centrifuge at 3000 rpm for 10 min at 4 °C. Supernatant was taken into autoclaved micro-centrifuge tube and stored at − 20 °C for further use. Western dot blot was performed to check cross reactivity of pre-immune serum with TLP. 200 µg of purified TLP was subcutaneously injected in two mice. TLP was emulsified with Freund's complete adjuvant (Sigma-Aldrich, USA) for first immunization, and for subsequent immunization (booster dosages), Freund's incomplete adjuvant (Sigma) was used. Antisera were collected 7 days after second and subsequent immunization and stored at − 20 °C for further use. Antisera were checked with dot blot to see reactivity of antisera with TLP.

Polyclonal antibody against ToLCNDV CP generated in rabbit was provided as a gift by Dr. V. G. Malathi (Adjunct Faculty, Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, India) and stored in small aliquots of 50 µL at − 20 °C for further use.

In vitro pull-down assay

In vitro pull-down was performed as previously performed by Rana et al. [7] with some modifications to check interaction of purified his-tagged TLP to GST-tagged CP of CLCuV and ToLCNDV in binding buffer [25 mM Tris–Cl (pH- 8), 5 mM $MgCl_2$, 75 mM NaCl, 2.5 mM DTT, 2.5 mM EDTA, and 1% NP40] in separate experiments. Pre-equilibrated 10 µL Glutathione Sepharose beads with binding buffer were added in each mixture and were incubated at 37 °C for 2 h with mild shaking. Samples were centrifuged at 4000 rpm for 3 min and unbound protein fractions were separated. Beads with bound proteins were washed twice with washing buffer [25 mM Tris–Cl (pH 8), 300 mM NaCl, 2.5 mM EDTA, 5 mM $MgCl_2$, and 0.1% NP40]. To bead bound fractions of proteins, 30 µL of SDS-sample buffer was mixed, whereas to unbound fraction 10 µL of SDS-sample buffer was mixed and boiled at 100 °C for 15 min. After short spin, 20 µL of supernatant was analyzed by 12% SDS-PAGE. Protein bands were observed by coomassie blue staining. Another in vitro pull-down assay was performed to revalidate interaction of TLP with CP of CLCuV and ToLCNDV. Western blot was developed using anti-TLP antibody generated in mouse, ALP-labeled anti-mouse antibody, and BCIP®/NBT substrate (Sigma). Experiment was performed in three independent replicates.

Dot blot assay

Interaction of TLP with purified ToLCNDV particles was performed using dot blot assay. ToLCNDV particles were purified from infected tomato leaves [35] and blotted on the nitrocellulose membrane. Membrane was blocked with 3% BSA in 1% PBS for 1 h at room temperature. Membrane was incubated with purified TLP (1/15) for 1 h at RT and then was washed twice with 1% PBST (1% PBS + 0.05% Tween 20). Blot was developed with primary anti-TLP antibody, ALP-labeled secondary anti-mouse antibody, and BCIP®/NBT substrate. For the negative control, no TLP incubation was performed with one blot. As a positive control, to confirm the presence of viral isolate one blot was developed with primary rabbit anti-ToLCNDV CP and secondary goat anti-rabbit antibodies.

Results

Yeast two-hybrid analysis

Yeast two-hybrid library screenings using cDNA of whole *B. tabaci* (as described in Materials and methods section) resulted in identification of various clones with specific candidate protein showing matches with various proteins

Table 2 List of best matching proteins upon BLASTx obtained from yeast two-hybrid libraries using ToLCNDV CP and CLCuV CP as bait

Clone names	Virus CP names	Best matching proteins upon BLASTx	Accession numbers[a]
TS4	ToLCNDV CP	*Bemisia tabaci* phosphatidylinositol-binding clathrin assembly protein LAP	XP_018914657
TSV36	ToLCNDV CP	*Bemisia tabaci* growth arrest and DNA-damage-inducible proteins-interacting protein 1	XP_018911056
CLCuV 54	CLCuV CP	*Bemisia tabaci* probable NADH dehydrogenase [ubiquinone] flavoprotein 2 (thioredoxin-like superfamily)	XP_018912341
CLCuV 45	CLCuV CP	*Bemisia tabaci* eukaryotic translation initiation factor 2 subunit 2	XP_018905510
CLCuV 37	CLCuV CP	*Bemisia tabaci* cytochrome c oxidase subunit II (mitochondrion)	AFK08574

[a]Accession numbers belong to best matching proteins upon BLASTx

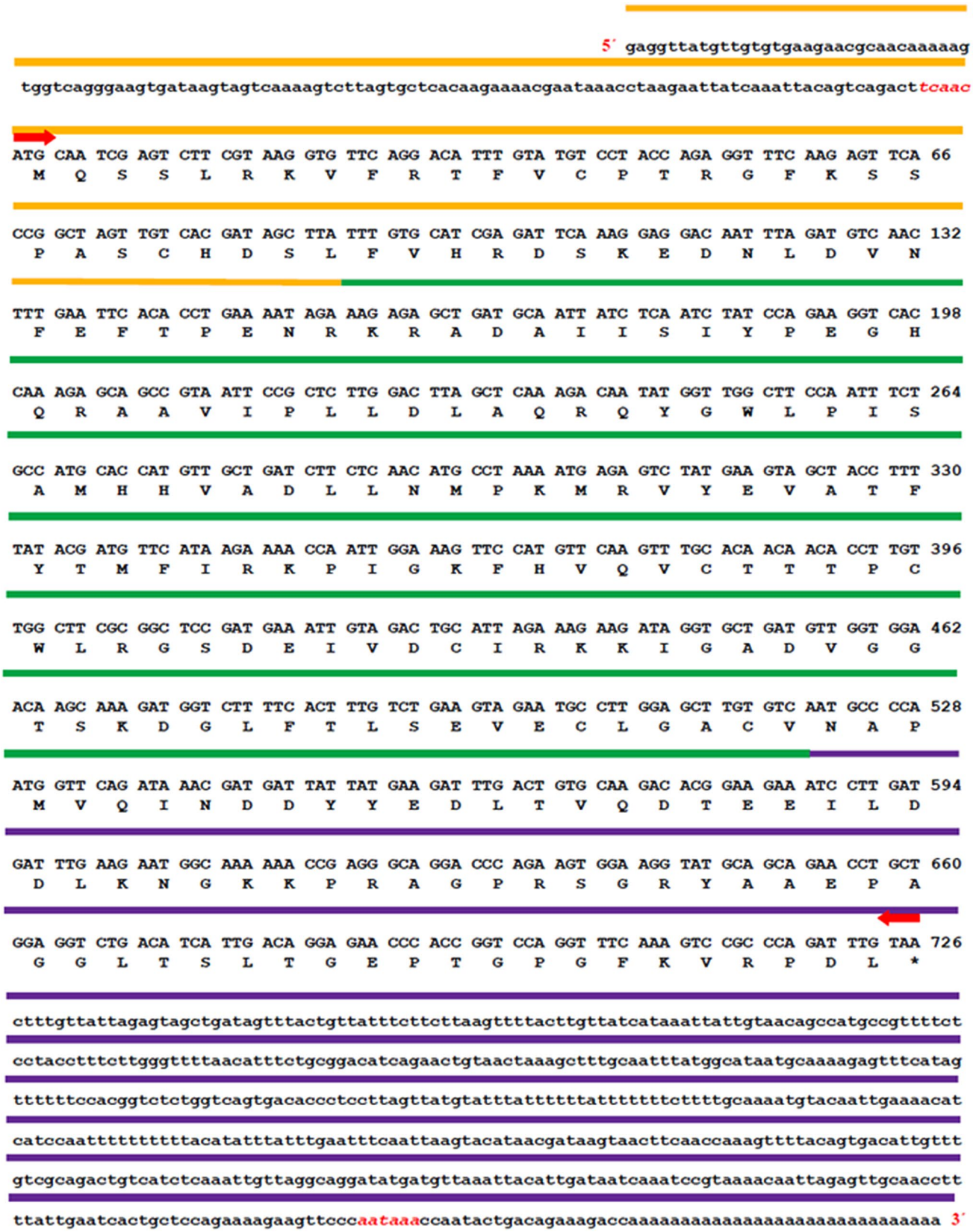
upon BLASTx (Table 2). Clone CLCuV 54 was selected for further work since this clone had a larger ORF. CLCuV 54 clone showed high similarity to thioredoxin-like superfamily of proteins and hence protein was named thioredoxin-like protein (TLP). Since TLP was identified from yeast two-hybrid library using CLCuV CP as bait, yeast two-hybrid assay followed by β-gal assay was performed to check in vivo interaction of TLP with ToLCNDV CP (Online Resource 1, Fig. S1). Plasmid construct used for this experiment is shown in Online Resource 1, Fig. S1. It can be noticed that in negative control, yeast cells could grow in 2DO media, but were unable to grow in 4DO media and also did not show any β-gal activity. In both positive control and experimental, transformed yeast cell colonies were able to grow in 4DO media and also showed β-gal activity, indicating that TLP has a positive interaction with the CP of ToLCNDV (Online Resource 1, Fig. S1).

5′ RACE and sequence analysis

Sequencing of the TLP clone from yeast two-hybrid library screening indicated the presence of an incomplete *tlp* ORF of 570-bp-long and 516-bp-long 3′ UTR followed by polyadenyl chain (Fig. 1). To complete the ORF, 5′ RACE was performed with primers, AAP, AUAP, TLP-R1, and TLP-R2 (Table 1), which resulted in the complete ORF and 5′ UTR (Fig. 1). There was a potential putative transcription initiator sequence (tcaac) and a possible polyadenylation signal (aataaa) in 5′ UTR and 3′ UTR, respectively (Fig. 1). Sequence analysis using BLASTn and BLASTx revealed that the 726-bp-long *tlp* gene could code for 241 amino acid long protein (Fig. 1). This sequence showed 99% identity with *B. tabaci* probable NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial (accession number XM_019056796). BLASTp analysis revealed that TLP showed 100% identity with *B. tabaci* probable NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial (Protein ID XP_018912341) of *B. tabaci*, which belonged to thioredoxin-like superfamily of proteins (Online Resource 1, Fig. S2). SignalP and ProtIdent indicated the absence of any signal peptide and protease property in TLP, respectively. RaptorX was used for protein structure model visualization (Online Resource 1, Figs. S3, S4). Secondary structure analysis revealed 35% helix, 7% beta sheet, and 56% coil structure in the TLP protein (Online Resource 1, Fig. S3). 3D structure was predicted by RaptorX using 5xtbO (cryo-EM structure of human respiratory complex I matrix arm) and 6g72E (mouse mitochondrial complex I in the de-active state) available in protein data bank as template with *p* value; 3.00e−07, uGDT (global distance test; GDT); 183 (76); and score of 184 (Online Resource 1, Fig. S4). These values suggested a good quality of structure prediction.

Cloning, expression, and purification of TLP and CP

Full-length *tlp* gene was cloned into a TA cloning vector, pGEM®-T Easy. Cloning was confirmed by performing restriction digestion (of positive clones after blue/white colony screening) with *Nde1* and *Sal1* restriction enzyme (Online Resource 1, Fig. S5) and reconfirmed by sequencing. Full-length *tlp* gene was submitted to NCBI GenBank with accession number KX865085. Plasmid (pGEM®-T Easy with *tlp* gene) was further restriction digested and *tlp* gene was sub-cloned into pET-28a expression vector using *Nde1* and *Sal1* restriction sites. *E. coli* DH5α was transformed with plasmid (pET-28a with *tlp* gene) and positively transformed cells were confirmed with restriction digestion using *Nde1* and *Sal1* (Online Resource 1, Fig. S6) and by sequencing. Sequence analysis confirmed the presence of proper gene frame and absence of any mutations. Later plasmid (pET-28a with *tlp* gene insert) was isolated and *E. coli* BL21 cells were transformed for TLP heterologous expression. TLP protein was first induced in 10 mL culture with 0.5 mM IPTG induction, which was checked on SDS-PAGE gel (Online Resource 1, Fig. S7). Later larger amount culture (300 mL) was used for protein induction; induced protein in soluble fraction was purified by using Ni–NTA beads and eluted in different fractions (Online Resource 1, Fig. S8). Eluted TLP fractions were pooled and dialyzed and stored at −70 °C till further use. Genes of CLCuV CP and ToLCNDV CP were previously cloned in pGEX-4T1 expression vector. *E. coli* strain {*Rosetta-gami 2*(*DE3*)*pLysS*} was transformed


5′ gaggttatgttgtgtgaagaacgcaacaaaaag
tggtcagggaagtgataagtagtcaaaagtcttagtgctcacaagaaaacgaataaacctaagaattatcaaattacagtcagacttcaac
ATG CAA TCG AGT CTT CGT AAG GTG TTC AGG ACA TTT GTA TGT CCT ACC AGA GGT TTC AAG AGT TCA 66
M Q S S L R K V F R T F V C P T R G F K S S
CCG GCT AGT TGT CAC GAT AGC TTA TTT GTG CAT CGA GAT TCA AAG GAG GAC AAT TTA GAT GTC AAC 132
P A S C H D S L F V H R D S K E D N L D V N
TTT GAA TTC ACA CCT GAA AAT AGA AAG AGA GCT GAT GCA ATT ATC TCA ATC TAT CCA GAA GGT CAC 198
F E F T P E N R K R A D A I I S I Y P E G H
CAA AGA GCA GCC GTA ATT CCG CTC TTG GAC TTA GCT CAA AGA CAA TAT GGT TGG CTT CCA ATT TCT 264
Q R A A V I P L L D L A Q R Q Y G W L P I S
GCC ATG CAC CAT GTT GCT GAT CTT CTC AAC ATG CCT AAA ATG AGA GTC TAT GAA GTA GCT ACC TTT 330
A M H H V A D L L N M P K M R V Y E V A T F
TAT ACG ATG TTC ATA AGA AAA CCA ATT GGA AAG TTC CAT GTT CAA GTT TGC ACA ACA ACA CCT TGT 396
Y T M F I R K P I G K F H V Q V C T T T P C
TGG CTT CGC GGC TCC GAT GAA ATT GTA GAC TGC ATT AGA AAG AAG ATA GGT GCT GAT GTT GGT GGA 462
W L R G S D E I V D C I R K K I G A D V G G
ACA AGC AAA GAT GGT CTT TTC ACT TTG TCT GAA GTA GAA TGC CTT GGA GCT TGT GTC AAT GCC CCA 528
T S K D G L F T L S E V E C L G A C V N A P
ATG GTT CAG ATA AAC GAT GAT TAT TAT GAA GAT TTG ACT GTG CAA GAC ACG GAA GAA ATC CTT GAT 594
M V Q I N D D Y Y E D L T V Q D T E E I L D
GAT TTG AAG AAT GGC AAA AAA CCG AGG GCA GGA CCC AGA AGT GGA AGG TAT GCA GCA GAA CCT GCT 660
D L K N G K K P R A G P R S G R Y A A E P A
GGA GGT CTG ACA TCA TTG ACA GGA GAA CCC ACC GGT CCA GGT TTC AAA GTC CGC CCA GAT TTG TAA 726
G G L T S L T G E P T G P G F K V R P D L *
ctttgttattagagtagctgatagtttactgttatttcttcttaagttttacttgttatcataaattattgtaacagccatgccgttttct
cctacctttcttgggttttaacatttctgcggacatcagaactgtaactaaagctttgcaatttatggcataatgcaaaagagtttcatag
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gtcgcagactgtcatctcaaattgttaggcaggatatgatgttaaattacattgataatcaaatccgtaaaacaattagagttgcaacctt
ttattgaatcactgctccagaaaagaagttcccaataaaccaatactgacagaaagaccaaaaaaaaaaaaaaaaaaaaaaaaaaaaa 3′

◂**Fig. 1** Complete *tlp* gene with 5′ and 3′ UTR. The 5′ and 3′ UTR are represented in lowercase. Potential putative transcription initiator sequence (tcaac) and possible polyadenylation signal (aataaa) are italic and in red color. Orange bar indicates sequence from 5′ RACE, green bar indicates sequence present in both 5′ RACE and yeast two-hybrid library, and purple color bar indicates sequence from yeast two-hybrid library. ORF boundary is indicated by red arrow from both sides

by these vectors with their respective gene and cultures were stored as glycerol stocks [7, 21]. Glycerol stocks of both were revived and induced with 0.5 mM IPTG (Online Resource 1, Fig. S7) and CPs of both CLCuV and ToLCNDV were isolated and purified for further experiments.

Generation and purification of anti-TLP antibody

BALB/c mice were used for generating antibody against TLP. Pre-immune sera when tested for cross reactivity showed no cross reactivity against TLP. 200 µg of TLP was used for immunization. Antisera were collected 7 days post second and subsequent immunizations. Antisera tested positive against TLP protein in dot blots (Online Resource 1, Fig. S9). Antisera were stored at − 20 °C for later use.

TLP interaction with CP of both CLCuV and ToLCNDV

The earlier results of yeast two-hybrid, wherein TLP interacted with CP of both CLCuV and ToLCNDV, were validated by in vitro pull-down assay. Result of the in vitro pull-down experiment indicated interaction of His-tagged TLP with GST-tagged CP of both CLCuV and ToLCNDV (Figs. 2, 3). In first control (with GST protein, but without CP), GST protein was observed in bound fraction but not TLP, whereas in second control (without both CP and GST proteins), no TLP was observed in bound fraction (Figs. 2, 3). These results suggested a specific interaction of TLP with CP of both CLCuV and ToLCNDV. Similar in vitro pull-down experiment was performed and western blot was developed as mentioned earlier (Fig. 4). Western blot revalidated that TLP interacts with CP of both CLCuV and ToLCNDV.

TLP interaction with ToLCNDV particles isolated from tomato leaves

Dot blot assay was performed to check interaction of TLP with purified ToLCNDV particles isolated from infected tomato leaves. When blotted ToLCNDV particles were allowed to interact with TLP and anti-TLP antibody was used as primary antibody, positive signal was observed. But in negative control, where TLP was not taken and blot was developed using anti-TLP antibody as primary antibody, no signal was observed. In positive control, blotted ToLCNDV particles were not incubated with TLP and blot was developed using anti-ToLCNDV antibody as primary antibody, signal was observed (Fig. 5). These results indicated that TLP interacted with ToLCNDV particles isolated from the infected tomato leaves.

Discussion

Begomoviruses are exclusively transmitted by *B. tabaci* in a persistent and circulative manner from infected plants to healthy plants [13]. During this type of transmission, begomoviruses traverse through stylet, esophagus, filter chamber, midgut, hemolymph, and salivary glands before being transmitted to the next healthy plant [14, 15]. Midgut epithelium acts as the main selective barrier in the transmission process in the insect vector [36]. Few studies have suggested that during transmission, begomoviruses interact with various proteins in the alimentary canal, filter chamber, midgut, hemolymph, and salivary glands of insect vector [19–23]. CP is the only viral protein which is reported to interact with proteins of the insect vector and is the determinant for the viral specificity in the insect vector [16–18]. To understand the complete pathway, identification of various interacting protein partners for the begomoviruses in the body of insect vector is essential and is yet to be performed comprehensibly.

Yeast two-hybrid assay is an effective and less expensive experiment to identify a novel protein–protein interaction partner in vivo. In this assay, cDNA acts as a prey and a particular protein acts as a bait, to find its possible interacting protein partners. In few studies, CP of begomoviruses was used as bait and cDNA library of *B. tabaci* was used as a prey to find possible interacting protein partners of CP inside insect vector [20, 21]. In this study, CPs of both CLCuV and ToLCNDV were used as bait and cDNA prepared from total RNA of whole body of *B. tabaci* was used as prey in different sets of experiments. Results from yeast two-hybrid cDNA screening led to identification of few possible interacting protein partners from both sets of experiments (Table 2), when sequences obtained were analyzed using BLASTn and BLASTx in NCBI server. The first candidate protein that we decided to work with in this study was clone CLCuV 54 (Table 2) since it had large ORF with complete 3′ UTR, which made complete generation of ORF along with 5′ UTR relatively easier by using 5′ RACE. This protein was named thioredoxin-like protein (TLP) since it belongs to thioredoxin-like superfamily of protein. Later, yeast two-hybrid assay using CP of ToLCNDV as bait confirmed that TLP also interacted with CP of ToLCNDV. TLP interaction with CP of both CLCuV and ToLCNDV prompted us to continue work on this clone. Complete gene sequence was generated using 5′ RACE (Fig. 1). BLASTn and BLASTp

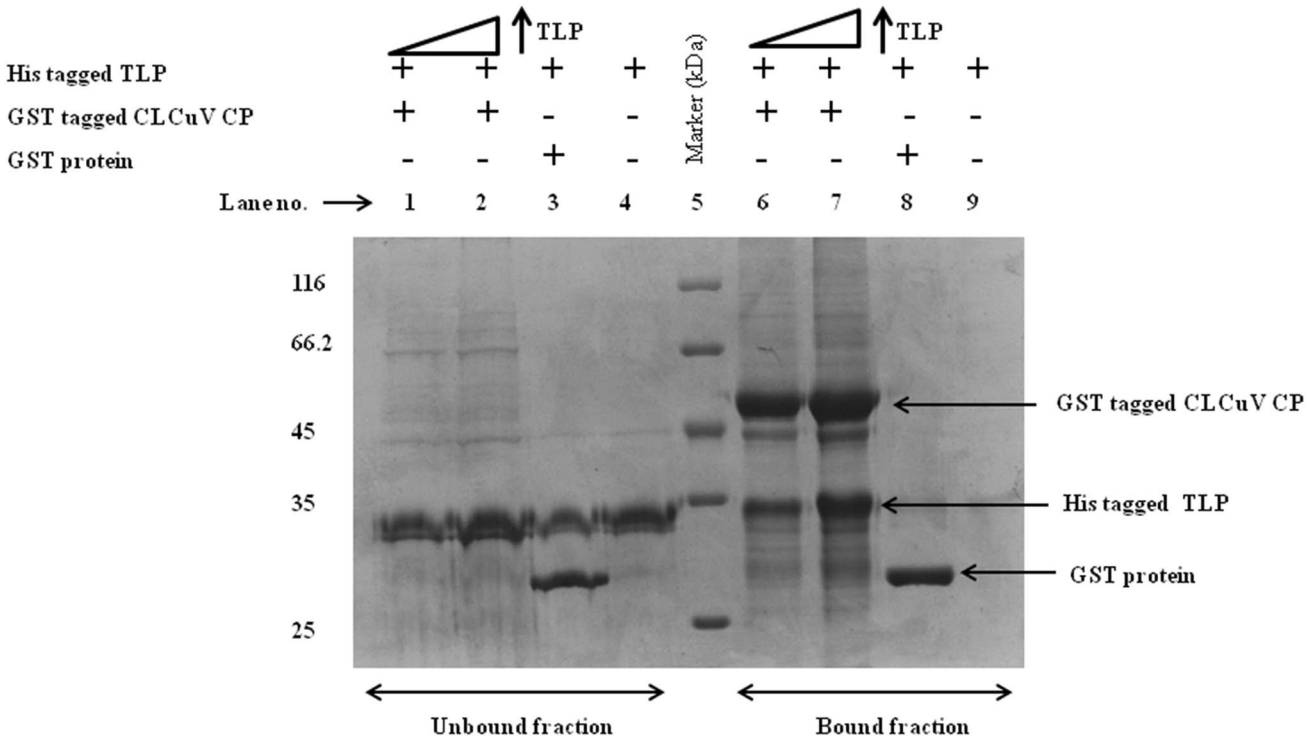

Fig. 2 In vitro pull-down assay to check interaction of His-tagged TLP with GST-tagged CLCuV CP. *Lanes 1* to *4* unbound fraction of proteins. *Lanes 6* to *9* bound fraction of proteins. *Lane 5* protein marker (kDa). *Lanes 1, 2, 6*, and *7* TLP and CLCuV CP were present, but *Lanes 2* and *7* has double amount of TLP than *Lanes 1* and *6*. *Lanes 3* and *8* TLP and GST proteins were present. *Lanes 4* and *9* only TLP was present

analyses showed similarity with *B. tabaci* probable NADH dehydrogenase (ubiquinone) flavoprotein 2, mitochondrial which belonged to thioredoxin-like superfamily of proteins (Online Resource 1, Fig. S2). Mitochondria have been found to play central role in host defense mechanisms against virus infections. A number of mitochondrial proteins and viral proteins have been found to be involved in the process viral infection. Some of viral protein have been localized in mitochondria and found to interact with mitochondrial proteins to regulate cellular processes in their favour [37]. In an earlier study, *Tomato yellow leaf curl China virus* (TYLCCNV) infection in *B. tabaci* was found to change the gene expression profile of the insect. One gene which showed homology to NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 5 was found to be up-regulated, whereas another gene, which showed homology to cytochrome b (CYTB) gene; NADH dehydrogenase subunit 1 (ND1) was down-regulated during the TYLCCNV infection [38].

Amino acid analysis of TLP indicated the absence of any signal peptide and protease property, thus hinting towards cytosolic nature of this protein. Earlier thioredoxins have been found to associate with cell organellae (mitochondria and nucleus) or found to be cytosolic or even extracellular [39]. TLP was found to interact with CP of both CLCuV and ToLCNDV in in vitro pull-down experiments. Further TLP was also found to interact with the ToLCNDV virus particles isolated from the tomato leaves in dot blot assay. Thus, yeast two-hybrid experiments, in vitro pull-down experiments, and the dot blot assay validated and confirmed the specific interaction of TLP with the CP of both CLCuV and ToLCNDV. Thioredoxin system has been discovered in many organisms (bacteria, yeast, plants, mammals etc.) and is ubiquitous in nature from archaea to humans [39]. Thioredoxins have been found to play various important roles in diverse organisms. Thioredoxins (ubiquitous proteins) are known to reduce disulfide bonds of many enzymes, which are present in subcellular compartment and are required in various biochemical reactions [40]. Thioredoxins are involved in redox regulation of protein function, cell signaling, in stress response, and pathogen–host interaction [39, 41, 42]. Thioredoxin is the only host protein in *E. coli* required for phage assembly and export [39, 43]. In *Zea mays*, ZmTrxh (an atypical thioredoxin protein) was found to work like molecular chaperon to confer resistance against *Sugarcane mosaic virus* (SCMV) in the early stage of infection [42]. Thus, interaction of TLP (a thioredoxin-like superfamily protein) with CP of both CLCuV and ToLCNDV might be involved in important function in begomoviral transmission by *B. tabaci*. Various proteins from *B. tabaci* have been identified to interact with the CP of begomoviruses and found to play important

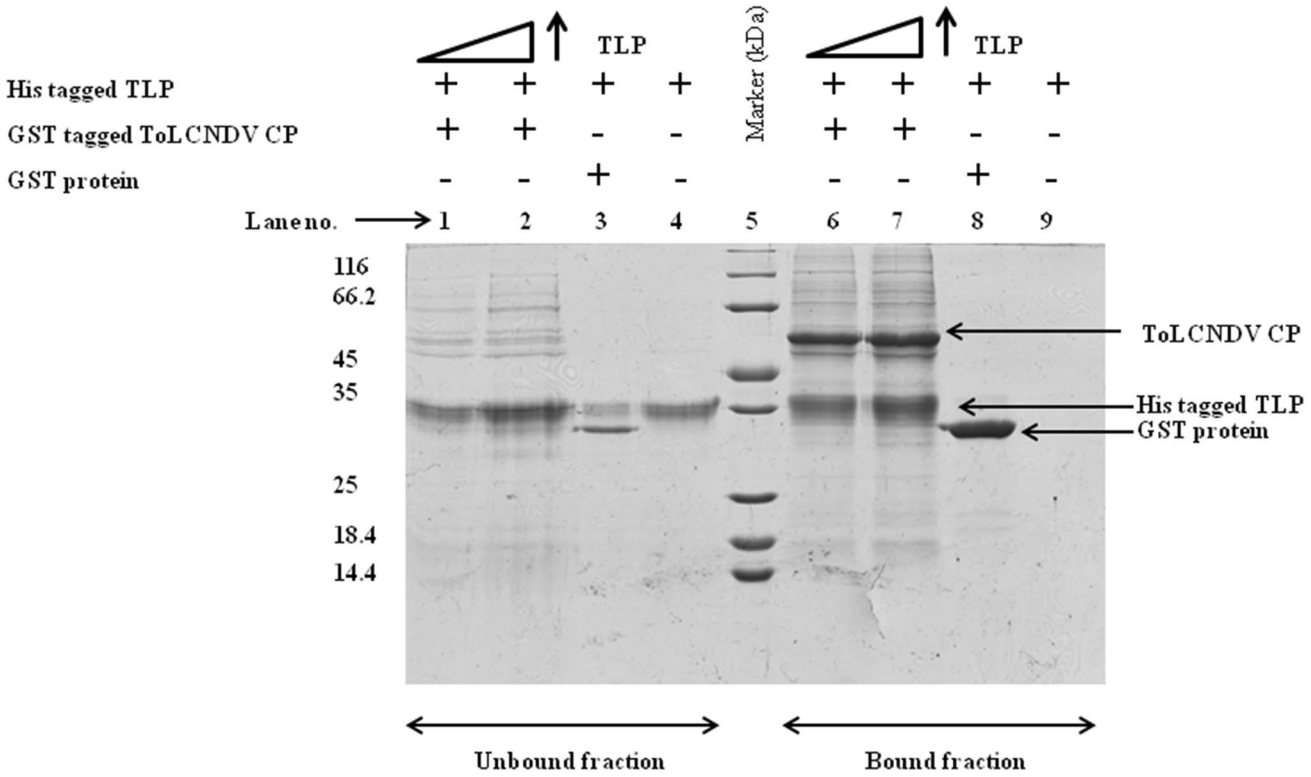

Fig. 3 In vitro pull-down assay to check interaction of His-tagged TLP with GST-tagged ToLCNDV CP. *Lanes 1* to *4* unbound fraction of proteins. *Lanes 6* to *9* bound fraction of proteins. *Lane 5* protein marker (kDa). *Lanes 1, 2, 6* and *7* TLP and ToLCNDV CP were present, but *Lanes 2* and *7* has double amount of TLP than *Lanes 1* and *6*. *Lanes 3* and *8* TLP and GST proteins were present. *Lanes 4* and *9* only TLP was present

role in the virus transmission. *B. tabaci* 16 kDa heat shock protein (BtHSP16) was identified as possible interacting partner of CP of *Tomato leaf curl Sardinia virus* (TYLCSV) by yeast two-hybrid assay and interaction was validated by in vitro pull-down assay [20]. A 70 kDa heat shock protein was identified to interact with CP of begomovirus and to play important roles in the begomoviral transmission [19]. In another study, MGP was observed to interact with the ToLCV in midgut and filter chamber and played important role in the virus acquisition and transmission [21]. Role of *knottin-1* gene in the transmission of TYLCV by *B. tabaci* was determined by dsRNA feeding [23]. Cyclophilin B has been reported to play important roles in the begomoviral transmission [22]. *B. tabaci* peptidoglycan recognition protein (BtPGRP) has a potential binding site for TYCLV [24]. Another study suggested the role of clathrin-mediated endocytosis in TYLCV transmission [25]. But complete picture of virus transmission by *B. tabaci* is still not clear.

In conclusion, we identified TLP which interacts with CP of both CLCuV and ToLCNDV. TLP also interacted with ToLCNDV particles isolated from tomato leaves. The precise role of TLP in the virus transmission and its accurate localization in *B. tabaci* need to be further investigated using different methodologies.

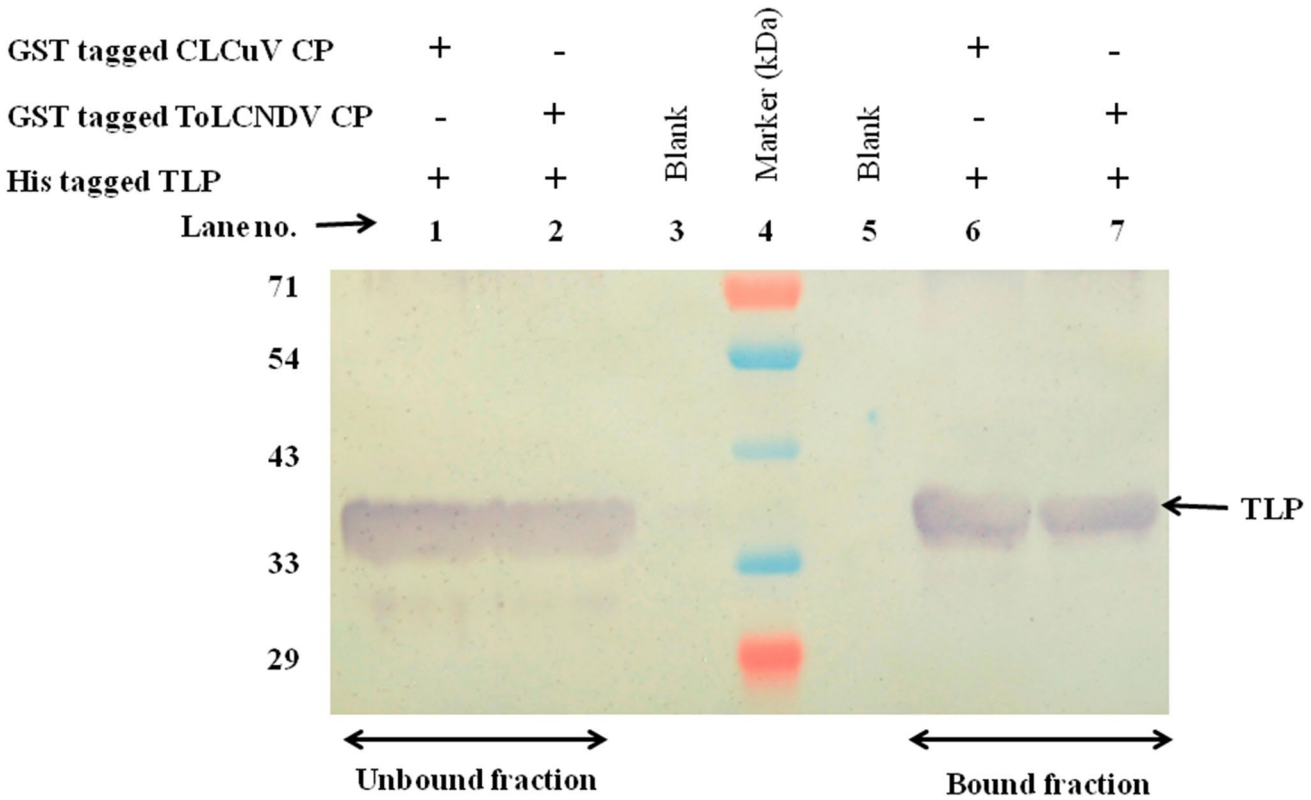

Fig. 4 Western blot of in vitro pull-down assay to revalidate interaction of His-tagged TLP with GST-tagged CLCuV CP and ToLCNDV CP. *Lanes 1* and *2* unbound fraction of proteins. *Lanes 6* and *7* bound fraction of proteins. *Lanes 3* and *5* blank. *Lane 4* protein marker (kDa). *Lanes 1* and *6* TLP and CLCuV CP were present. *Lanes 2* and *7* TLP and ToLCNDV CP were present

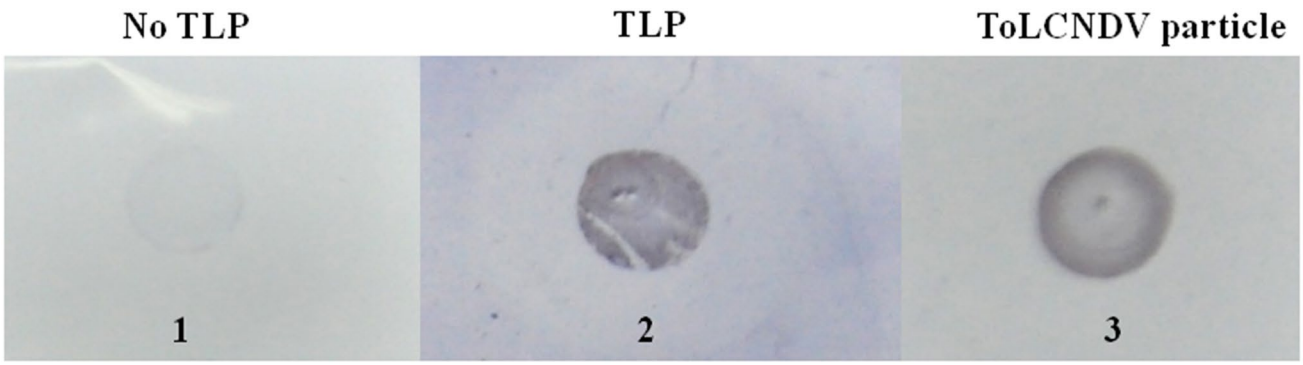

Fig. 5 Dot blot assay to check interaction of TLP with the ToLCNDV particles. (1) *Negative control* no TLP was used to interact with the blotted ToLCNDV particles. (2) TLP protein was allowed to interact with the blotted ToLCNDV particles. In both 1 and 2, blots were developed with anti-TLP antibody. (3) *Positive control* TLP was not used for interaction with the blotted ToLCNDV particles and blot was developed with anti-ToLCNDV CP antibody

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