

ORIGINAL ARTICLE

FKBP-type peptidyl-prolyl *cis-trans* isomerase interacts with the movement protein of tomato leaf curl New Delhi virus and impacts viral replication in *Nicotiana benthamiana*

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Ministry of Science and Technology, Taiwan, Grant/Award Number: 105-2313-B-005-019-MY3 and 108-2313-B-005-034-MY3.

Abstract

Begomoviruses belonging to the family *Geminiviridae* are plant-infecting DNA viruses. Begomoviral movement protein (MP) has been reported to be required for virus movement, host range determination, and symptom development. In the present study, the FK506-binding protein (FKBP)-type peptidyl-prolyl *cis-trans* isomerase (NbFKPPIase) of *Nicotiana benthamiana* was identified by a yeast two-hybrid screening system using the MP of tomato leaf curl New Delhi virus (ToLCNDV) oriental melon (OM) isolate (MP^{OM}) as bait. Transient silencing of the gene encoding NbFKPPIase increased replication of three test begomoviruses, and transient overexpression decreased viral replication, indicating that NbFKPPIase plays a role in defence against begomoviruses. However, infection of *N. benthamiana* by ToLCNDV-OM or overexpression of the gene encoding MP^{OM} drastically reduced the expression of the gene encoding NbFKPPIase. Fluorescence resonance energy transfer analysis revealed that MP^{OM} interacted with NbFKPPIase in the periphery of cells. Expression of the gene encoding NbFKPPIase was induced by salicylic acid but not by methyl jasmonate or ethylene. Moreover, the expression of the gene encoding NbFKPPIase was down-regulated in response to 6-benzylaminopurine and up-regulated in response to gibberellin or indole-3-acetic acid, suggesting a role of NbFKPPIase in plant development. Transcriptome analysis and comparison of *N. benthamiana* transient silencing and overexpression of the gene encoding MP^{OM} led to the identification of several differentially expressed genes whose functions are probably associated with cell cycle regulation. Our results indicate that begomoviruses could suppress NbFKPPIase-mediated defence and biological functions by transcriptional inhibition and physical interaction between MP and NbFKPPIase to facilitate infection.

KEYWORDSbegomovirus, movement protein, peptidyl-prolyl *cis-trans* isomerase, replication, tomato leaf curl New Delhi virus (ToLCNDV)

This work was also financially supported (in part) from the framework of the Higher Education Sprout Project by the Ministry of Education, Taiwan.

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1 | INTRODUCTION

Viruses in the *Geminiviridae* family can infect a broad range of monocotyledonous and dicotyledonous plants and cause devastating damage to crops in tropical and subtropical regions. The incidence and severity of diseases caused by geminiviruses, often transmitted by whiteflies, have increased considerably in the past 20 years (Mansoor et al., 2006; Navas-Castillo et al., 2011). Geminiviruses have a small single-stranded DNA genome consisting of a circular monopartite (DNA-A-like) or bipartite (DNA-A and DNA-B) genome encoding five to seven proteins (Zerbini et al., 2017).

Tomato leaf curl New Delhi virus (ToLCNDV) is a bipartite begomovirus infecting plants in the Solanaceae and Cucurbitaceae families (Hussain et al., 2005; Khan et al., 2012; López et al., 2015; Padidam et al., 1995; Samretwanich et al., 2000; Usharani et al., 2004). Most ToLCNDV isolates are not mechanically transmissible to their host plants. However, a ToLCNDV oriental melon (OM) isolate, originally collected from a diseased oriental melon plant, can be mechanically transmissible (Chang et al., 2010; Tsai et al., 2011), and its mechanical transmissibility has been demonstrated to be associated with the movement protein (MP) of viral DNA-B (Lee et al., 2020). Viral MP is a multifunctional protein involved in cell-to-cell and systemic movement of viruses (Krenz et al., 2012). MP is involved in begomoviral intracellular trafficking and indirectly cooperates with nuclear shuttle protein (NSP) to facilitate nuclear entry of begomoviruses (Happle et al., 2021; Noueiry et al., 1994). MP has been reported to be a determinant of host range and a virulence factor (Garrido-Ramirez et al., 2000; Ingham & Lazarowitz, 1993; Ingham et al., 1995).

Geminiviral proteins must interact with a wide range of plant proteins and rely on host cellular functions to facilitate their replication and cell-to-cell movement (Hanley-Bowdoin et al., 2013). Both replication enhancer protein and replication initiator protein of begomoviruses have been demonstrated to collaboratively interact with proliferating cell nuclear antigen and suppress the retinoblastoma-related protein of host plants, thus reprogramming the cell cycle and facilitating viral replication (Settlage et al., 2005). The nuclear shuttle protein (NSP) of begomoviruses is considered to be involved in the intracellular trafficking of viral DNAs. To date, more evidence seems to indicate that NSPs interact with host factors not only for nucleus-cytoplasm shuttling but also for plant defence suppression. NSPs interact with acetyltransferase and GTPase (NIG) to facilitate viral DNA transportation (Carvalho & Lazarowitz, 2004; Carvalho et al., 2008; McGarry et al., 2003). NSP also interacts with an endogenous gene silencing suppressor (AS2) and a transmembrane leucine-rich repeat receptor-like kinase (NIK1) to suppress host defence (Fontes et al., 2004; Mariano et al., 2004; Ye et al., 2015).

Geminiviral MP has been shown to interact with host proteins to facilitate the transport of viral DNA into the nucleus and enhance viral movement and infectivity. MP of bean dwarf mosaic virus (MP^{BDMV}) interacts with the histone H3 protein, forming a minichromosome to facilitate inter- and intracellular transport of viral DNA (Zhou et al., 2011). MP of Abutilon mosaic virus (MP^{AbMV}) interacts with cHSP70,

facilitating cell-to-cell movement (Krenz et al., 2010). MP of cabbage leaf curl virus (MP^{CaLCuV}) interacts with synaptotagmin A (SYTA) to facilitate virus movement through plasma membrane-derived endosomes to plasmodesmata (Lewis & Lazarowitz, 2010). Suppressing the expression of the genes encoding cHSP70 or SYTA significantly delays viral infection and symptom development, strongly demonstrating the requirement of MP-host protein interactions for viral pathogenesis. *Nicotiana benthamiana* stomatal cytokinesis defective 2, a cytokinesis and expansion protein, interacts with MP^{AbMV}, and this interaction might be involved in virus transport, similar to SYTA (Krapp et al., 2017). In addition to movement function, MP^{AbMV} interacts with *N. benthamiana* peptidyl-prolyl *cis-trans* isomerase NIMA-interacting 4 (Pin4), influences cell cycle regulation, and enables virus replication (Krapp et al., 2017). Based on the abovementioned studies, the notion that begomoviral proteins interact with host plant proteins that alter host DNA synthesis and the defence system and reprogramme the host cell proliferation system to favour viral replication and movement was well supported.

An FK506-binding protein (FKBP)-type peptidyl-prolyl *cis-trans* isomerase (NFKPPIase) of *N. benthamiana* was identified by a yeast two-hybrid (Y2H) assay using the MP of ToLCNDV-OM (MP^{OM}) as bait. The peptidyl-prolyl *cis-trans* isomerase (PPIase) superfamily consists of a group of proteins that have the ability to catalyse *cis-trans* isomerization of proteins containing phosphoserine/threonine-proline motifs in all organisms (Nath & Isakov, 2015). PPIases are classified into four subfamilies according to their affinities to immunosuppressive drugs, cyclophilins (Cyps), FKBP, parvulins, and chimeric proteins with FK506- and cyclosporine-binding parts. PPIases play a critical role in cell cycle regulation, signal transduction, protein secretion, developmental processes, and host-pathogen interactions (Kromina et al., 2008). The PPIase Pin1 has been implicated in the regulation of immune cell functions and other cellular processes associated with ageing, cancer, and Alzheimer's disease (Lee et al., 2011; Liou et al., 2011; Nath & Isakov, 2015; Yeh & Means, 2007). Cyclophilins have been demonstrated to be required for viral replication in animals (Frausto et al., 2013). CypA interacts with a Gag protein of human immunodeficiency virus 1 and enhances viral infectivity (Braaten & Luban, 2001). Human CypB interacts with the nonstructural protein 5B of hepatitis C virus and facilitates viral replication in humans (Watashi et al., 2005). Cyclophilins have been shown to play a critical role in the replication of several RNA viruses, including influenza A and nidovirus (Liu et al., 2012; de Wilde et al., 2018). A study of the interaction between tomato bushy stunt virus and plants has shown that CypA binds to viral RNA and replication proteins and inhibits viral replication in yeast and plant leaves (Kovalev & Nagy, 2013). In plants, the FKBP-type PPIases act as molecular chaperones to interact with other proteins to modulate a wide range of developmental processes, stress responses, and plant defences (Gollan et al., 2012; Kurek et al., 1999; Mokryakova et al., 2014; Xiong et al., 2016). *Arabidopsis* FKBP12 (AtFKBP12) has been reported to be involved in the target of rapamycin (TOR) protein signalling pathway (John et al., 2011). TOR protein complexes act

as a central hub in the signalling pathway and modify several downstream proteins by phosphorylation. The TOR-mediated signalling pathway modulates diverse cellular processes, including autophagy, protein translation, DNA methylation, and cell cycle regulation (Burkart & Brandizzi, 2020). AtFKBP12 senses rapamycin, forming a ternary complex, and inhibits the activity of TOR proteins. *Arabidopsis* Pin4 has been reported to interact with MP^{AbMV} for cell cycle regulation (Krapp et al., 2017). The role of plant PPlases in viral replication and infection remains largely unknown.

In the present study, an NbFKPPlase was identified and demonstrated to interact with MP^{OM}. Transient silencing and overexpression analyses revealed that NbFKPPlase may play a role in plant defence. However, begomoviruses could overcome the NbFKPPlase-mediated defence system by suppressing the expression of the gene encoding NbFKPPlase and by a direct interaction between MP^{OM} and NbFKPPlase, thus effectively increasing viral replication.

2 | RESULTS

2.1 | Identification of plant proteins interacting with MP^{OM} by Y2H

A cDNA library of *N. benthamiana* was constructed and screened by the Y2H system using MP^{OM} as bait, which resulted in the recovery of 452 clones on SD/Leu⁻/Trp⁻/His⁻/X-Gal medium. Plasmids were individually purified and retransformed into yeast, which led to the identification of 94 clones that enabled yeast to grow on SD/Leu⁻/Trp⁻/His⁻ medium. All clones were sequenced. Database similarity searching coupled with Gene Ontology (GO) analysis revealed their putative protein functions, which could be categorized into 26 groups. These groups included proteins associated with translation (ribosomal proteins), photosystem or function in chloroplasts (oxygen-evolving enhancer proteins), protein folding (HSP20-like protein, chaperonin Cpn10, and peptidyl-prolyl *cis-trans* isomerase), nucleotide-binding functions (glycine-rich RNA-binding protein and chromodomain helicase DNA-binding protein), signal transduction (annexin D4), and metabolic pathways (Table 1).

2.2 | FKBP-type peptidyl-prolyl *cis-trans* isomerase suppresses ToLCNDV-OM replication

Transient gene silencing analyses were conducted to identify gene products that are probably involved in ToLCNDV-OM infection. In total, nine clones, namely, oxygen-evolving enhancer protein 1, ferredoxin, glycine-rich RNA-binding protein 3, annexin D4, Kunitz trypsin inhibitor 1, peptidyl-prolyl *cis-trans* isomerase, 30S ribosomal protein S5, hypothetical mitotic cell cycle regulator (CDY16734.1), and SYTA, were analysed by transient gene silencing. *N. benthamiana* plants were pre-infected with ToLCNDV-OM. Agrobacteria

carrying a hairpin silencing construct were infiltrated into a leaf with symptoms 8 days after viral inoculation. DNA was purified and examined by quantitative PCR using ToLCNDV-OM coat protein primers 5 days after agro-infiltration. The results revealed that infiltration of agrobacteria carrying a hairpin silencing construct of the gene encoding PPlase increased the accumulation of ToLCNDV-OM in comparison to the hairpin silencing construct of phytoene desaturase (hpPDS), which was used as a negative control (Figure 1a). Infiltration of hairpin silencing constructs of the other eight clones failed to change ToLCNDV-OM accumulation (data not shown).

Sequencing analysis of the gene encoding full-length PPlase (Sol Genomics Network database accession number Niben101Scf07001g01011) indicated that the gene encodes a 12-kDa polypeptide containing an FK506-binding domain belonging to the FKBP-type PPlase protein family and was herein designated NbFKPPlase. The NbFKPPlase coding region was cloned and used for overexpression assays to further confirm its biological function. Transient overexpression of the gene encoding NbFKPPlase in *N. benthamiana* reduced the accumulation of ToLCNDV-OM compared to the plants expressing green fluorescent protein (GFP) (Figure 1b).

2.3 | NbFKPPlase plays a role in the replication of begomoviruses

Transient gene silencing and overexpression of the gene encoding NbFKPPlase in *N. benthamiana* were performed to test its effects on two other begomoviruses: a cucumber strain of ToLCNDV (ToLCNDV-CB) and tomato yellow leaf curl Thailand virus (TYLCTHV). Transient silencing of the gene encoding NbFKPPlase increased DNA accumulation of ToLCNDV-CB (Figure 1c), and overexpression of the gene encoding NbFKPPlase suppressed ToLCNDV-CB replication (Figure 1d). Similar results were observed in *N. benthamiana* inoculated with TYLCTHV. Transient silencing of the gene encoding NbFKPPlase increased TYLCTHV replication (Figure 1e), and overexpression of the gene encoding NbFKPPlase suppressed its replication (Figure 1f).

2.4 | Bimolecular fluorescence complementation and fluorescence resonance energy transfer analyses confirm in planta interactions between MP^{OM} and NbFKPPlase

To further verify the interactions between NbFKPPlase and MP^{OM}, bimolecular fluorescence complementation (BiFC) analysis was conducted. Yellow fluorescent protein (YFP) signals were observed at the cell periphery in all combinations of YFP reconstitution constructs (data not shown). BiFC combinations containing the N- or C-terminal part of YFP showed stronger YFP signals when fused to the C-terminus of MP^{OM} than when fused to the N-terminus (Figure S1). However, BiFC analysis revealed only the NbFKPPlase-MP^{OM} interaction position. Fluorescence

TABLE 1 Putative movement protein interaction partners identified by yeast two-hybrid screening and Gene Ontology analysis

Gene	Accession number ^a	GO term ^b	Description ^c
Protein folding			
HSP20-like protein	Niben101Scf02513g00006.1, Niben101Scf02877g02002.1, Niben101Scf02437g06011.1, Niben101Scf01412g00015.1		
Chaperonin Cpn10	Niben101Scf08651g04006.1	GO:0005737, GO:0006457, GO:0005524	(CC) cytoplasm; (BP) protein folding; (MF) ATP binding
Peptidyl-prolyl <i>cis-trans</i> isomerase	Niben101Scf07001g01011.1	GO:0006457	(BP) protein folding
Chloroplast/ photosystem			
Oxygen-evolving enhancer protein 1	Niben101Scf05555g00008.1, Niben101Scf05943g00002.1	GO:0016021, GO:0042549, GO:0019898, GO:0005509, GO:0009279, GO:0009654	(CC) integral component of membrane; (BP) photosystem II stabilization; (CC) extrinsic component of membrane; (MF) calcium ion binding; (CC) cell outer membrane; (CC) photosystem II oxygen-evolving complex
Oxygen-evolving enhancer protein 2	Niben101Scf06826g08015.1	GO:0009654, GO:0015979, GO:0005509, GO:0019898	(CC) photosystem II oxygen-evolving complex; (BP) photosynthesis; (MF) calcium ion binding; (CC) extrinsic component of membrane
Synaptotagmin A	Niben101Scf07109g01003.1	GO:0005515	(MF) protein binding
Translation			
30S ribosomal protein S5	Niben101Scf01436g03010.1	GO:0003735, GO:0006412, GO:0015935, GO:0003723	(MF) structural constituent of ribosome; (BP) translation; (CC) small ribosomal subunit; (MF) RNA binding
30S ribosomal protein S10	Niben101Scf05581g00006.1	GO:0003735, GO:0006412, GO:0005840, GO:0003723	(MF) structural constituent of ribosome; (BP) translation; (CC) ribosome; (MF) RNA binding
60S ribosomal protein L23a	Niben101Scf01444g02009.1	GO:0003735, GO:0006412, GO:0005840, GO:0000166	(MF) structural constituent of ribosome; (BP) translation; (CC) ribosome; (MF) nucleotide binding
50S ribosomal protein L14	Niben101Scf01892g01001.1	GO:0003735, GO:0015934, GO:0006412	(MF) structural constituent of ribosome; (CC) large ribosomal subunit; (BP) translation
Nucleotide binding			
Glycine-rich RNA-binding protein 3	Niben101Scf00757g00001.1, Niben101Scf05279g01002.1	GO:0003676, GO:0000166	(MF) nucleic acid binding; (MF) nucleotide binding
Chromodomain helicase DNA-binding protein	Niben101Scf02026g08001.1	GO:0005524, GO:0003677	(MF) ATP binding; (MF) DNA binding
Other biological processes			
2-dehydro-3-deoxy-phosphoheptonate aldolase	Niben101Scf01450g00005.1, Niben101Scf02537g04006.1, Niben101Scf11865g01003.1	GO:0003849, GO:0009073	(MF) 3-deoxy-7-phosphoheptulonate synthase activity; (BP) aromatic amino acid family biosynthetic process
Annexin D4	Niben101Scf02792g02012.1	GO:0005544, GO:0005509	(MF) calcium-dependent phospholipid binding; (MF) calcium ion binding
Proteasome subunit β 1	Niben101Scf11609g01012.1	GO:0051603, GO:0005839, GO:0004298	(BP) proteolysis involved in cellular protein catabolic process; (CC) proteasome core complex; (MF) threonine-type endopeptidase activity

TABLE 1 (Continued)

Gene	Accession number ^a	GO term ^b	Description ^c
Iron-sulphur cluster assembly protein	Niben101Scf00472g06014.1	GO:0005506, GO:0051536, GO:0016226	(MF) iron ion binding; (MF) iron-sulphur cluster binding; (BP) iron-sulphur cluster assembly
Pentatricopeptide repeat-containing protein	Niben101Scf02354g04005.1	GO:0005515	(MF) protein binding
12-oxophytodienoate reductase 3	Niben101Scf02467g01003.1	GO:0016491 GO:0055114 GO:0010181	(MF) oxidoreductase activity; (BP) oxidation-reduction process; (MF) flavin mononucleotide (FMN) binding
Ferredoxin	Niben101Scf22688g00018.1	GO:0022900 GO:0016021 GO:0051537 GO:0009055	(BP) electron transport chain; (CC) integral component of membrane; (MF) 2 iron, 2 sulphur cluster binding; (MF) electron transfer activity
Kunitz trypsin inhibitor 1	Niben101Scf01971g01005.1	GO:0004866	(MF) endopeptidase inhibitor activity
Cysteine-rich extensin-like protein	Niben101Scf09417g02001.1		
Actin	Niben101Scf00096g04015.1		
CDY16734.1	Niben101Scf08873g00007.1		
NADH dehydrogenase 1 β subcomplex subunit 8	Niben101Scf08640g00003.1		
Phosphorylated carbohydrates phosphatase	NCBI XM_019386734.1		
Zinc finger protein	Niben101Scf01094g01010.1		

^aAccession number from the Sol Genomics Network (<https://solgenomics.net/>) or the NCBI (<https://www.ncbi.nlm.nih.gov/>) database.

^bGene Ontology analysis was conducted in the AgriGO (<http://systemsbiology.cau.edu.cn/agriGOv2/index.php>) database.

^cMF, molecular function; CC, cellular component; BP, biological process.

resonance energy transfer (FRET) analysis was conducted to illustrate the location of individual proteins and interaction positions. Based on the BiFC results, the YFP sequence was fused to the C-terminus of MP^{OM} to produce a pKMP-YFP clone, and the cyan fluorescent protein (CFP) sequence was fused to the C-terminus of NbFKPPlase to produce a pKPPI-CFP clone. Agrobacteria containing pKCFP-PPI and pKMP-YFP clones were co-expressed in *N. benthamiana* leaves after agro-infiltration. Three days after agro-infiltration, leaves were collected for fluorescence observation (Figure 2). Cyan fluorescence (excitation 405 nm/emission 460–500 nm) emitted from the NbFKPPlase:CFP fusion protein was observed widely in the periphery of the cells. Yellow fluorescence (excitation 488 nm/emission 530–630 nm) emitted from the MP^{OM}:YFP fusion protein was also observed in the cells, particularly along the periphery of the cells. Yellow fluorescence was observed using FRET channels (excitation 405 nm/emission 530–630 nm), indicating the interaction between NbFKPPlase:CFP and MP^{OM}:YFP. Based on the pattern of fluorescence, interactions between the two proteins could occur on the cell membrane, and the signal among different cells showed similar fluorescence strength. Comparing the fluorescence obtained from virus background

samples, the interactions between the two proteins were also observed. However, the fluorescence of some cells with stronger NbFKPPlase:CFP signals showed weaker MP^{OM}:YFP signals, and some cells showed the opposite patterns (Figure 2).

2.5 | ToLCNDV-OM suppresses the expression of the gene encoding NbFKPPlase

Reverse transcription quantitative PCR (RT-qPCR) was performed to examine the transcript level of the gene encoding NbFKPPlase in *N. benthamiana* leaves with or without ToLCNDV-OM challenge. The results indicated that the expression of the gene encoding NbFKPPlase was significantly down-regulated in *N. benthamiana* after infection with ToLCNDV-OM compared to the plants treated with buffer only (Figure 3a). RT-qPCR analysis of the ToLCNDV-OM coat protein gene confirmed viral replication. Transient overexpression of MP^{OM} in *N. benthamiana* significantly decreased the transcript level of the gene encoding NbFKPPlase compared to the plants expressing GFP (Figure 3b), indicating that MP^{OM} could affect NbFKPPlase gene expression.

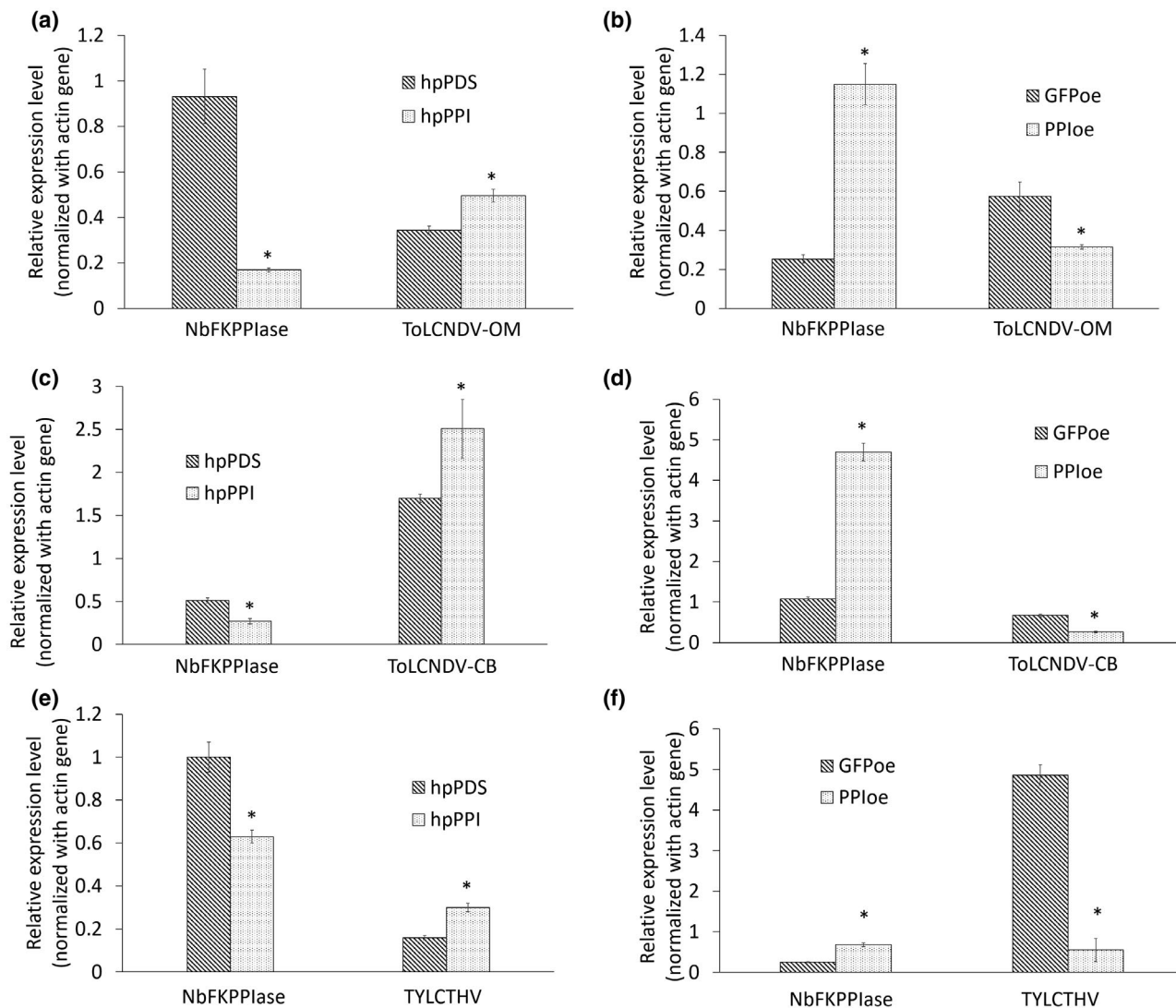


FIGURE 1 FKBP-type peptidyl-prolyl *cis-trans* isomerase (NbFKPPIase) plays a role in the replication of begomoviruses in *Nicotiana benthamiana*. Expression of the gene encoding NbFKPPIase was examined by reverse transcription quantitative PCR, and expression of the genes encoding coat proteins of begomoviruses was examined by quantitative PCR. *N. benthamiana* leaves were infected with tomato leaf curl New Delhi virus oriental melon isolate (ToLCNDV-OM) (a,b), CB isolate (ToLCNDV-CB) (c,d), or tomato yellow leaf curl Thailand virus (TYLCTHV) (e,f). After 8 days, leaves were infiltrated with agrobacteria carrying the NbFKPPIase hairpin silencing construct (pK7-hpPPI; hpPPI) or the NbFKPPIase overexpression construct (pK2-PPI; PP1oe). Leaves infiltrated with agrobacteria carrying pK7-hpPDS (hpPDS) and pK2-GFP (GFPoe) were used as negative controls for silencing and overexpression, respectively. Data are presented as mean \pm SD. Asterisks (*) indicate significant differences ($p < 0.05$) compared to the control. Expression of the *actin* gene was used as an internal control for normalization

2.6 | Expression of the gene encoding NbFKPPIase is impacted by phytohormones

RT-qPCR was performed to examine the transcript level of the gene encoding NbFKPPIase in *N. benthamiana* leaves after being treated with salicylic acid (SA), methyl jasmonate (JA), or 1-aminocyclopropane-1-carboxylic acid (an ethylene precursor, hereafter referred to as ET). The results indicated that the transcript levels of the gene encoding NbFKPPIase increased 3 h after SA treatment and gradually decreased 6 h after treatment, showing similar patterns in the expression of the gene encoding PR1 (Figure 4a). Expression of a protodermal factor 2.1 (PDF2.1)

gene implicated in the JA signalling pathway and an ethylene-responsive element-binding protein (EREBP) gene was up-regulated by JA and ET, respectively (Figure 4b,c). Expression of the gene encoding NbFKPPIase was not affected by JA and ET treatments. RT-qPCR analyses revealed that transient silencing or overexpression of the gene encoding NbFKPPIase in *N. benthamiana* apparently had no impact on the expression of the genes encoding NPR1 or PR1 (Figure 4d,e). However, the expression of the gene encoding NbFKPPIase in *N. benthamiana* seedlings was down-regulated after 6-benzylaminopurine (BA) treatment and up-regulated after gibberellin (GA) or indole-3-acetic acid (IAA) treatment (Figure 4f).

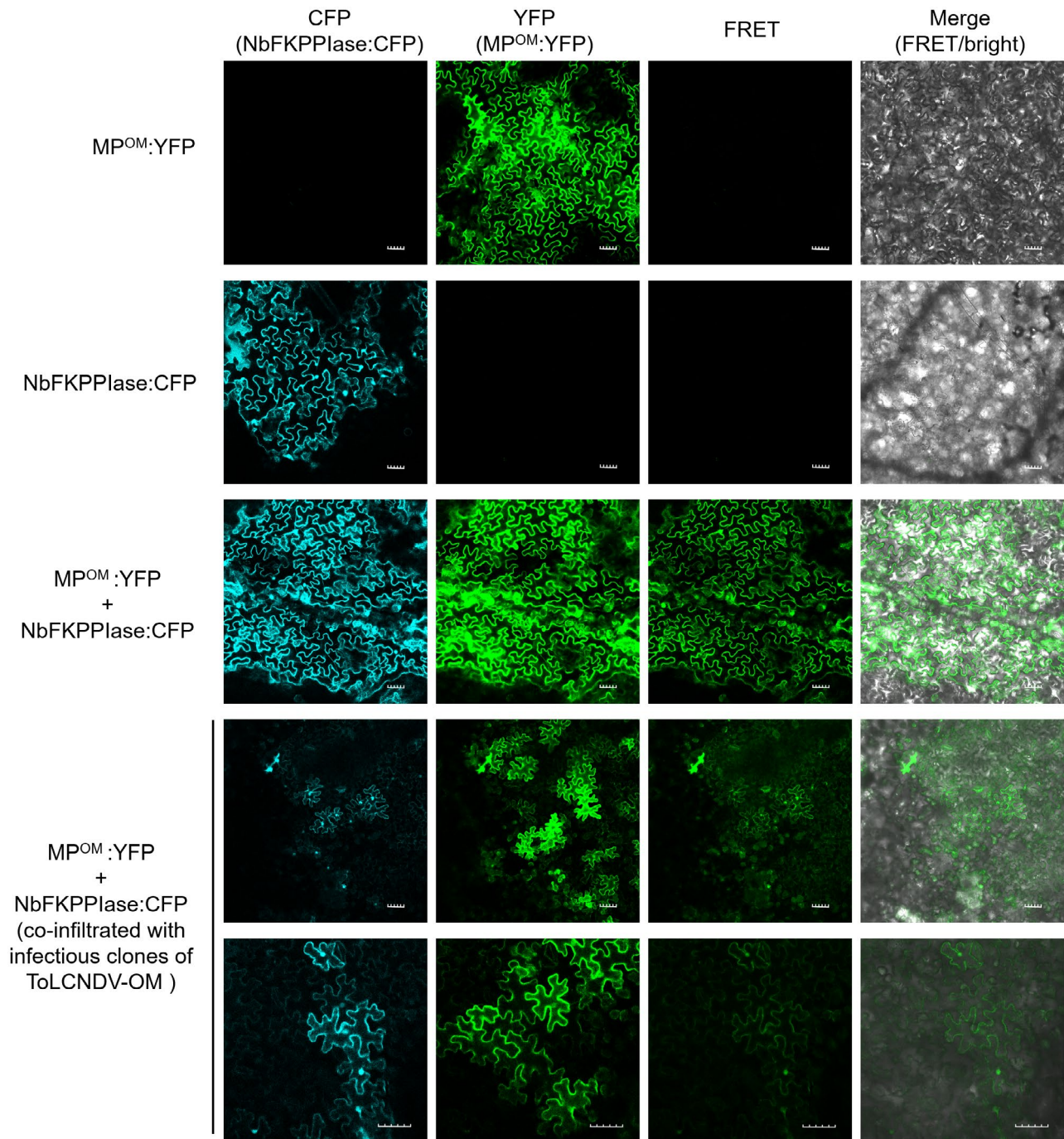


FIGURE 2 Fluorescence resonance energy transfer (FRET) analysis confirms in planta interactions between ToLCNDV-OM MP (MP^{OM}) and FKBP-type peptidyl-prolyl *cis-trans* isomerase (NbFKPPlase). Agrobacteria carrying pKPI-CFP (expressing PPIase:CFP fusion protein) and pKMP-YFP (expressing MP^{OM}:YFP fusion protein) were individually or co-infiltrated into *Nicotiana benthamiana* leaves. Agrobacteria carrying pKPI-CFP and pKMP-YFP were also co-infiltrated with infectious clones of ToLCNDV-OM for virus background analysis. Three days postinfiltration, leaves were collected for fluorescence observation using an Olympus FV3000 confocal microscope equipped with different wavelength channels: CFP (excitation 405 nm/emission 460–500 nm), YFP (excitation 488 nm/emission 530–630 nm), FRET (excitation 405 nm/emission 530–630 nm), and bright field. Images were processed using FV31S-SW software. Scale bar = 50 μ m

2.7 | Transcriptome analysis reveals the involvement of NbFKPPlase in cell cycle regulation

RNA sequencing analysis was conducted by a Nanopore system (Oxford Nanopore Technology) to compare gene expression

profiles in *N. benthamiana* after transient silencing or overexpression of the gene encoding NbFKPPlase. As a result, 20,727 and 18,758 uniquely mapped reads were identified from transient silencing and overexpression samples, respectively. False discovery rate (FDR)-adjusted $p < 0.05$ was used to identify differentially

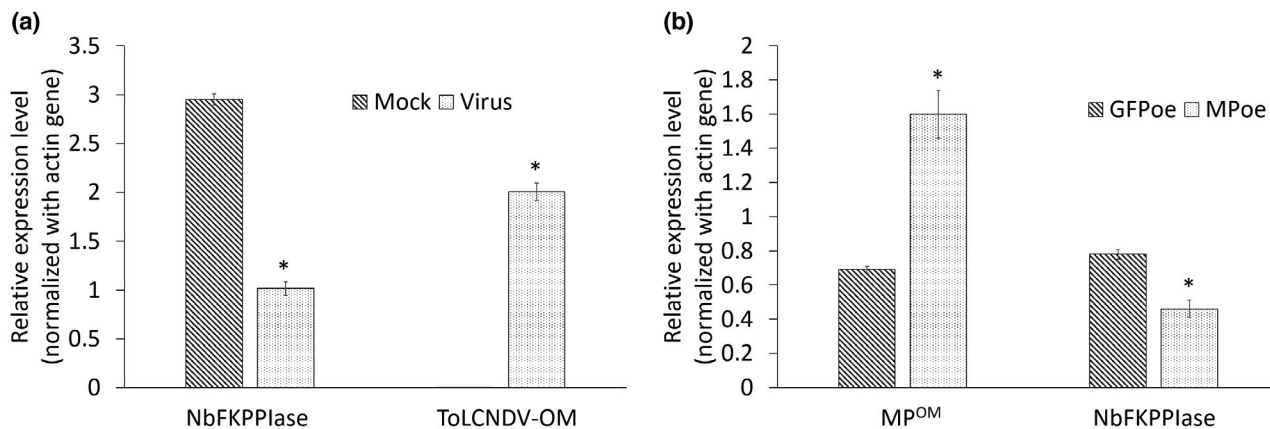


FIGURE 3 ToLCNDV-OM suppresses the expression of the gene encoding FKBP-type peptidyl-prolyl *cis-trans* isomerase (NbFKPPIase). (a) Reverse transcription quantitative PCR (RT-qPCR) was performed to examine the transcript level of the gene encoding NbFKPPIase in *Nicotiana benthamiana* plants challenged with buffer (Mock) or ToLCNDV-OM (Virus), revealing that ToLCNDV-OM suppresses the accumulation of transcripts encoding NbFKPPIase. Replication of ToLCNDV-OM was confirmed by quantitative PCR (qPCR) analysis of the coat protein-coding gene of DNA-A. (b) Reverse transcription-qPCR analysis revealed that transient overexpression of the ToLCNDV-OM movement protein (MPoe) in *N. benthamiana* decreased the transcript accumulation of the gene encoding NbFKPPIase compared to that in leaves transiently overexpressing GFP alone (GFPoe). Asterisks (*) indicate significant differences ($p < 0.05$) compared to the control. All data were normalized against the expression level of the *actin* gene

expressed genes (DEGs). Comparing transient silencing and overexpression samples, 3,508 up-regulated and 2,512 down-regulated genes were identified. GO analysis identified the top 15 GO annotation terms (Figure 5). Of them, five up-regulated and seven down-regulated genes were found to encode proteins that are probably involved in the cell cycle (Table 2). RT-qPCR analyses revealed that transient overexpression of the gene encoding MP^{OM} in *N. benthamiana* decreased the expression of the gene encoding NbFKPPIase (Figure 6a). Expression of the genes encoding a GTP-binding protein (Niben101Scf00682g02010.1), a cyclin-dependent kinase (Niben101Scf07965g04002.1), a Mis12 superfamily protein (Niben101Scf01661g02023.1), and a cyclin-dependent kinase regulatory subunit (Niben101Scf01464g01005.1) implicated in the cell cycle was up-regulated in *N. benthamiana* transiently overexpressing the gene encoding MP^{OM} of ToLCNDV-OM (Figure 6b). Expression of the genes encoding cell cycle-related cyclin superfamily proteins (Niben101Scf09153g02001.1 and Niben101Scf00163g15003) was down-regulated, however.

3 | DISCUSSION

Viral MP plays a wide range of roles during plant-virus interactions. MP^{BDMV} has been shown to interact with plant histone H3 protein in plasmodesmata, facilitating virus trafficking (Zhou et al., 2011). Both cHSP70 (Krenz et al., 2010) and SYTA (Lewis & Lazarowitz, 2010) have also been shown to interact with MP^{AbMV} and MP^{CaLCuV}, respectively, to facilitate cell-to-cell and systemic movement of viruses. MPs have been shown to be a determinant of host range and virulence in various viruses (Garrido-Ramirez et al., 2000; Ingham & Lazarowitz, 1993; Ingham et al., 1995). Several reports have indicated that begomovirus MP participates in movement and biological

process modulation. MP^{AbMV} may perpetuate the cellular S-phase by interacting with a Pin4 protein (Krapp et al., 2017). MP^{OM} has recently been demonstrated to be involved in the mechanical transmission of ToLCNDV-OM (Chang et al., 2010; Lee et al., 2020). In the current study, a number of plant proteins, including NbFKPPIase, actin, SYTA, and proteins with chaperonin functions (Table 1), from *N. benthamiana* were identified by a Y2H assay using MP^{OM} as bait. Transient silencing analyses revealed that oxygen-evolving enhancer protein 1, ferredoxin, glycine-rich RNA-binding protein 3, annexin D4, Kunitz trypsin inhibitor 1, 30S ribosomal protein S5, CDY16734.1, and SYTA play no roles in the accumulation of ToLCNDV-OM. Silencing of the gene encoding NbFKPPIase decreased ToLCNDV-OM accumulation, however.

The role of PPIases in viral replication has been studied in some detail in animals (Braaten & Luban, 2001; Watashi et al., 2005). The role of plant PPIases in viral infection is uncertain. Transient silencing analyses have demonstrated that NbFKPPIase plays a role in defence against begomovirus infection, as silencing of the gene encoding NbFKPPIase led to an increase in the replication of three different begomoviruses: ToLCNDV-OM, ToLCNDV-CB, and TYLCTHV. Conversely, transient overexpression of the gene decreased viral replication, further confirming the involvement of NbFKPPIase in plant defence. Despite its role in defence, begomoviruses apparently are able to overcome the defence function of NbFKPPIase by engaging in a direct interaction with viral MP. As demonstrated by FRET analyses, MP^{OM} could interact with NbFKPPIase in planta and led to an increase in virus accumulation. The interaction appears to occur in various cellular compartments, as shown in the periphery of cells based on the fluorescence pattern using a FRET channel. Compared to nonvirus conditions, some cells with stronger MP^{OM}:YFP signals showed weaker NbFKPPIase:CFP signals, and some cells showed opposite patterns under a virus co-infiltration background, which was

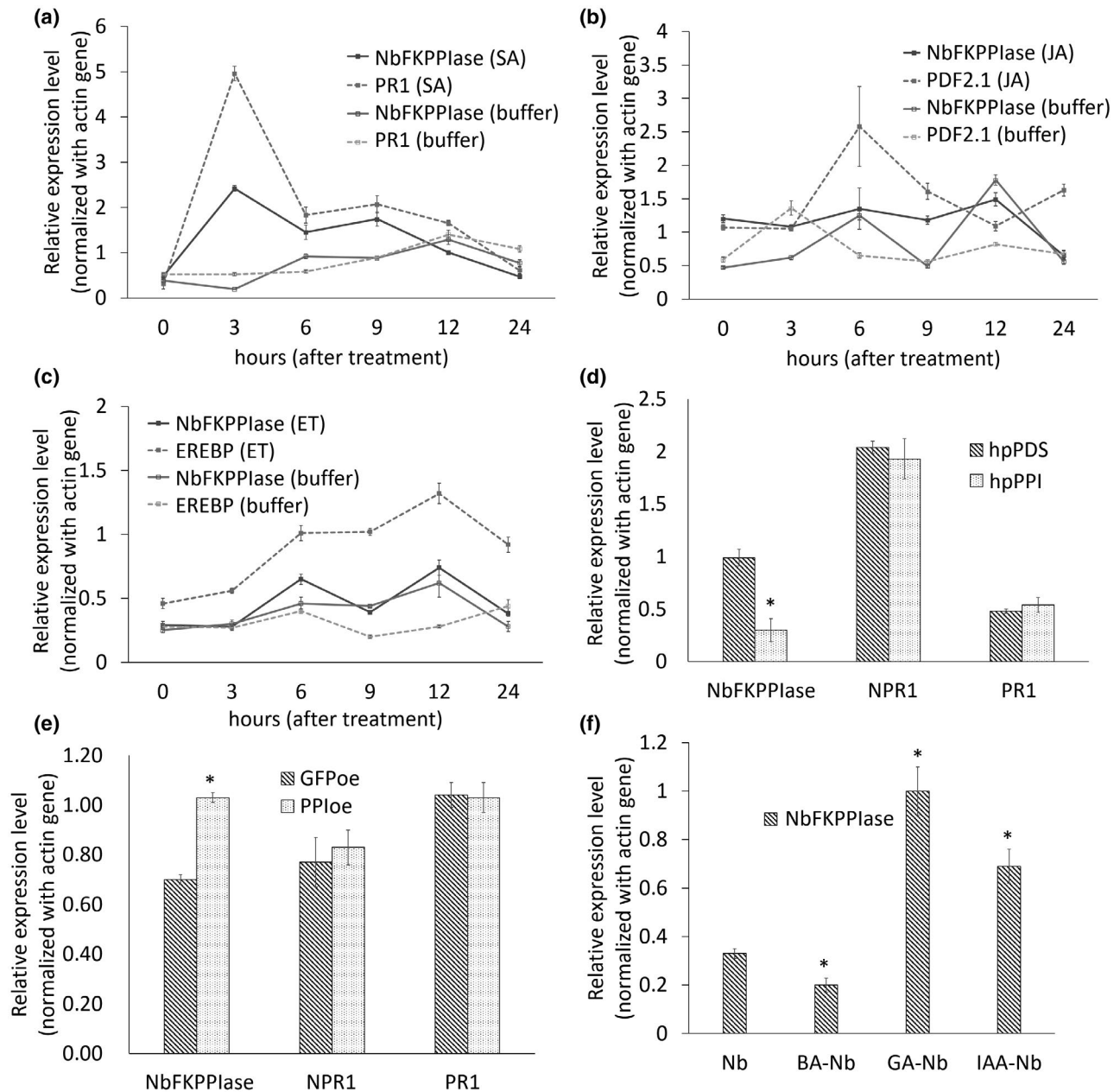


FIGURE 4 Expression of the gene encoding FKBP-type peptidyl-prolyl *cis-trans* isomerase (NbFKPPlase) is impacted by plant hormones. (a) Reverse transcription quantitative PCR (RT-qPCR) analysis of the genes encoding NbFKPPlase and PR1 in *Nicotiana benthamiana* leaves after treatment with salicylic acid (SA) or buffer (mock control) over time. (b) RT-qPCR analysis of the genes encoding NbFKPPlase and protodermal factor 2.1 (PDF2.1) in *N. benthamiana* leaves after treatment with methyl jasmonate (JA) or buffer. (c) RT-qPCR analysis of the genes encoding NbFKPPlase and ethylene-responsive element-binding protein (EREBP) in *N. benthamiana* leaves after treatment with 1-aminocyclopropane-1-carboxylic acid (ethylene precursor, ET) or buffer. (d) RT-qPCR analysis of the genes encoding NbFKPPlase, NPR1, and PR1 in *N. benthamiana* after infiltration with agrobacteria carrying the NbFKPPlase hairpin silencing construct (hpPPI). Leaves infiltrated with agrobacteria carrying hpPDS were used as the negative control. (e) RT-qPCR analysis of the genes encoding NbFKPPlase, NPR1, and PR1 in *N. benthamiana* after infiltration with agrobacteria carrying the NbFKPPlase overexpression construct (PPloe). Leaves infiltrated with agrobacteria carrying GFP alone (GFPoe) were used as the negative control. (f) RT-qPCR analysis of the gene encoding NbFKPPlase in *N. benthamiana* seedlings after being treated with 6-benzylaminopurine (BA), gibberellin (GA), or indole-3-acetic acid (IAA). Data are presented as mean \pm SD. Asterisks (*) indicate significant differences ($p < 0.05$) compared to the control. All data were normalized against the expression level of the *actin* gene

a surprising observation. Previous experiments showed that transient overexpression of the gene encoding NbFKPPlase decreased ToLCNDV-OM accumulation (Figure 1). ToLCNDV-OM infection and transient overexpression of the gene encoding MP^{OM} decreased the

expression level of the gene encoding NbFKPPlase (Figure 3). Thus, the gene expression levels of NbFKPPlase and MP^{OM} have a negative relationship. ToLCNDV-OM infection may stabilize and increase the accumulation of MP^{OM}, leading to decreased NbFKPPlase. Further

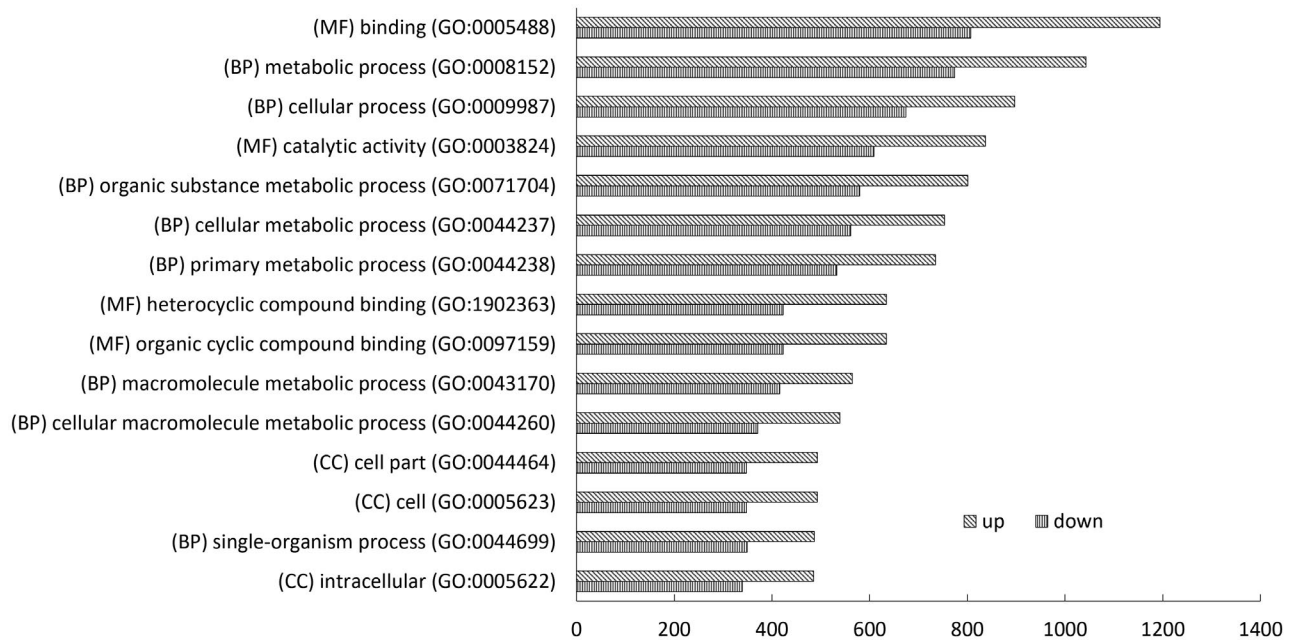


FIGURE 5 Transcriptome analysis reveals the involvement of FKBP-type peptidyl-prolyl *cis-trans* isomerase (NbFKPPlase) in a wide range of biological processes. RNA sequencing data of *Nicotiana benthamiana* after transient silencing or overexpression of the gene encoding NbFKPPlase were analysed. Gene Ontology (GO) analysis of identified differentially expressed genes was conducted in three separate categories: molecular function (MF), biological process (BP), and cellular component (CC). “up” indicates that genes were up-regulated and “down” indicates that genes were down-regulated when the gene encoding NbFKPPlase was transiently silenced

TABLE 2 Differentially expressed genes classified into the cellular component category of Gene Ontology (GO) terms in FKBP-type peptidyl-prolyl *cis-trans* isomerase (NbFKPPlase) transient silencing vs. transient overexpression transcriptome analysis

Accession number ^a	Weighted proportions fold change ^b	FDR-adjusted <i>p</i> -value	Description	Homologous gene in <i>Arabidopsis</i> ^c
Niben101Scf00682g02010.1	∞	0.0005	GTP-binding protein; may be involved in cell division	AT2G22870
Niben101Scf12585g00007.1	∞	0.0013	Microtubule-associated protein; may be involved in DNA replication, recombination, and repair	AT2G01910
Niben101Scf07965g04002.1	4.26	0.02	Cyclin-dependent kinase; may be involved in cell cycle regulation	AT5G27620
Niben101Scf01661g02023.1	3.41	0.02	Mis12 superfamily protein; plays a vital role in chromosome segregation	AT5G35520
Niben101Scf01464g01005.1	1.60	0.04	Cyclin-dependent kinase regulatory subunit	AT2G27960
Niben101Scf09153g02001.1	-∞	0.0023	Cyclin superfamily	AT3G21870
Niben101Scf24758g01007.1	-∞	0.0004	Hydroxyproline-rich glycoprotein family protein	None
Niben101Scf03398g06003.1	-∞	0.0006	Hydroxyproline-rich glycoprotein family protein	None
Niben101Scf18125g00014.1	-∞	0.01	Meiotic chromosome segregation	None
Niben101Scf07722g01003.1	-1.61	0.0002	Thioredoxin-like protein 4A	AT5G08290
Niben101Scf00801g04021.1	-2.94	0.02	Sequence-specific DNA-binding transcription factor activity; may be involved in cell cycle regulation	AT5G03415
Niben101Scf00163g15003.1	-1.76	0.05	Cyclin superfamily	AT2G44740

^aAccession number from the Sol Genomics Network (<https://solgenomics.net/>) database.

^bRatio of NbFKPPlase silencing: overexpression.

^cGene sequences were download from the Sol Genomics Network database and then analysed with BLAST on the *Arabidopsis* database (<https://www.arabidopsis.org/>).

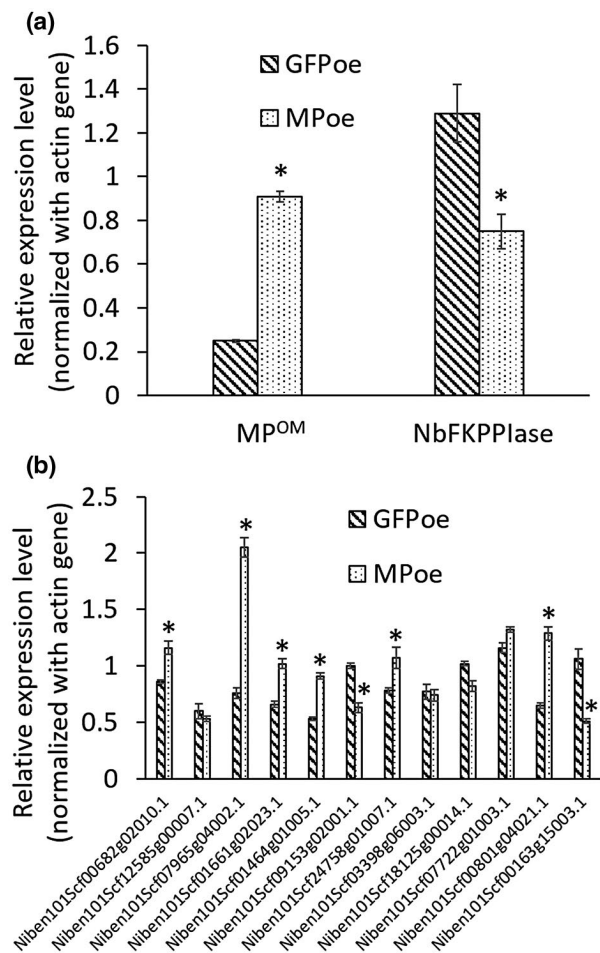


FIGURE 6 Identification of the genes involved in cell cycle regulation in *Nicotiana benthamiana* after transiently expressing the ToLCNDV-OM movement protein (*MP^{OM}*). (a) Reverse transcription quantitative PCR (RT-qPCR) analysis of the genes encoding *MP^{OM}* and *NbFKPPIase* in *N. benthamiana* transiently overexpressing *MP^{OM}* (*MPoe*) or GFP alone (*GFPoe*). (b) RT-qPCR analysis of the genes identified by transcriptome analysis of *N. benthamiana* transiently overexpressing *MP^{OM}* (*MPoe*) or GFP alone (*GFPoe*). Data are presented as mean \pm SD. Asterisks (*) indicate significant differences ($p < 0.05$) compared to the control. All data were normalized against the expression level of the *actin* gene

experiments are needed to clarify whether this protein–protein interaction leads to *NbFKPPIase* protein degradation.

To determine in which defence pathway *NbFKPPIase* may participate, the *NbFKPPIase* gene expression response to SA, JA, or ET treatment was analysed. The results indicated that expression of the gene encoding *NbFKPPIase* was induced by SA but not JA or ET. However, transient silencing or overexpression of the gene encoding *NbFKPPIase* in *N. benthamiana* apparently had no impact on the expression of the genes encoding *NPR1* or *PR1*. These results suggested that *NbFKPPIase*-mediated defence is not associated with *NPR1*-mediated plant defence. PPIase is a multifunctional protein involved in a wide range of developmental processes, stress responses, and plant defences (Gollan et al., 2012; Kurek et al., 1999; Mokryakova et al., 2014; Xiong et al., 2016). Vittorioso

et al. (1998) reported the involvement of an FKBP-type PPIase, encoded by the *PASTICCINO1* gene, in plant development and found that this gene was regulated by BA treatment. Further analyses revealed that the expression of the gene encoding *NbFKPPIase* was up-regulated by GA and IAA but down-regulated by BA. These results indicated that the *NbFKPPIase* identified in this study may be involved in cell cycle regulation. Thus, infection with begomoviruses, including ToLCNDV-OM, ToLCNDV-CB, and TYLCTHV, led to the suppression of the expression level of the gene encoding *NbFKPPIase* to reprogramme the host cells into the S-phase, which is similar to the observations after BA treatment.

Krapp et al. (2017) identified a Pin4 protein interacting with *MP^{AbMV}*. Tobacco rattle virus-based virus-induced gene silencing of the Pin4 gene enables infected tobacco plants to grow faster than nonsilenced plants. AbMV on Pin4-silenced plants also accumulated a higher viral DNA amount than nonsilenced plants. The Pin4 protein is a parvulin-like PPIase and its gene shares low (42.6%) nucleotide identity with the gene encoding *NbFKPPIase*. However, the Pin4 protein plays similar roles to *NbFKPPIase*, including interacting with begomoviral MP and being involved in cell cycle regulation yet benefitting viral DNA accumulation only when the Pin4 gene was silenced.

Transcriptome analyses of *N. benthamiana* transient silencing or overexpression of the gene encoding *NbFKPPIase* have led us to identify numerous DEGs. Many of these genes encode proteins with functions associated with cell cycle regulation, suggesting that *NbFKPPIase* also plays a critical role in the cell cycle. ToLCNDV-OM infection or *MP^{OM}* overexpression significantly decreased the expression of the gene encoding *NbFKPPIase*, indicating that ToLCNDV-OM affected *NbFKPPIase* at the transcriptional level. Although *NbFKPPIase* may play a role in plant defence, physical interactions between *NbFKPPIase* and viral MP coupled with transcriptional suppression may minimize its impact and allow begomoviruses to replicate efficiently. Begomoviruses can suppress plant defence genes or proteins via MP to modulate host biological processes and facilitate replication.

AtFKBP12 (AT5G64350) is the *Arabidopsis* orthologue of *NbFKPPIase*. AtFKBP12 has been reported as a bridge between rapamycin and TOR protein involved in the TOR signalling pathway. The TOR pathway modulates growth–defence trade-offs in plant stress responses (Margalha et al., 2019). The active form of the TOR complex activates cell cycle regulation (Burkart & Brandizzi, 2020). Upon binding to rapamycin, AtFKBP12 inhibits the activity of the TOR complex and blocks the cell cycle in the G1 phase (Vilella-Bach et al., 1999). *NbFKPPIase* shares 75.2% nucleotide identity and 79.6% amino acid identity with AtFKBP12. *NbFKPPIase* may play a similar role in the TOR signalling pathway in *N. benthamiana*. ToLCNDV-OM infection or a high expression level of *MP^{OM}* reduced *NbFKPPIase* expression. Low expression of *NbFKPPIase* led to activation of the TOR complex and activated related genes to process cell cycle regulation.

To the best of our knowledge, this is the first report of an FKBP-type PPIase interacting with viral MP in plants. The biological

relevance and mechanism of this interaction warrant further study. Our results indicate that while trafficking with viral DNA, MP could interact with host factors and influence biological processes to create a suitable environment for virus infection before expressing other viral proteins. Thus, the virus movement complex plays more complicated roles than simply being involved in virus movement.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plant materials and phytohormone treatment

The *N. benthamiana* plants used in this study were maintained in a greenhouse located at National Chung Hsing University (Taichung, Taiwan). Five-week-old leaves were sprayed with sodium salicylate (SA; 5 mM), JA (250 μ M), ET (2 mM), or water (mock control) then collected at varying times (0, 3, 6, 9, 12, and 24 hr) for RNA extraction and gene expression analysis. *N. benthamiana* seeds were germinated on half-strength Murashige & Skoog (MS) medium (2.2 g MS salts, 10 g sucrose, and 8 g bacterial agar in 1 L) amended with or without BA (1 mg/L), GA (0.25 mg/L), or IAA (0.5 mg/L). After 10 days, 15 seedlings were collected and pooled for RNA extraction and gene expression analysis.

4.2 | Purification of nucleic acids and PCR analysis

Plant RNA was extracted with TRIzol reagent (Ambion; Life Technologies). Samples were treated with RNase-free recombinant DNase I (Takara Bio Inc.) for 1 h to remove genomic DNA, extracted with phenol/chloroform, and precipitated with ethanol. For gene expression analysis, first-strand cDNA was synthesized from RNA (1 μ g) with oligo-dT primer (FJJ2003-46, 200 ng) and Moloney murine leukaemia virus (MMLV) reverse transcriptase following the manufacturer's instructions (Invitrogen Life Technologies). RT-qPCR was conducted to analyse gene expression. Reactions were set up using an iQ-SYBR Green Supermix reagent kit (Bio-Rad Laboratories) with cDNA product (50 ng) and carried out in a CFX Connect Real-time System (Bio-Rad). PCR amplification began by pre-incubating at 95°C for 3 min, followed by 40 cycles of 95°C (10 s) and 55°C (30 s). PCR products were sequenced to validate their identities. All genes were analysed from three biological replicates, and each sample contained three technical replicates. The relative quantification and statistical analysis were performed using CFX Maestro software (Bio-Rad) with one-way analysis of variance (ANOVA) and Tukey's test ($p < 0.05$). The *actin* gene was used as an internal control.

DNA was extracted from *N. benthamiana* plants as described previously (Lee et al., 2020) and used for PCR amplification with 25 ng of total DNA per reaction. The cycling programme was as follows: 95°C for 3 min, followed by 40 cycles of 95°C (10 s) and 58°C (30 s). The accumulation of DNA viruses was analysed by PCR with gene-specific primers. All data were normalized against the expression level of the *actin* gene. The primer pairs used in this study are listed in Table S1.

4.3 | Y2H screening

The full-length MP^{OM} sequence was amplified by PCR with the primer pair FJJ2014-22/FJJ2014-23 (Table S1). The amplicon was cloned into a pGBK vector (Clontech) fused with the N-terminal GAL4 DNA-binding domain to produce a pGBKOMMP plasmid as a bait vector. pGBKOMMP was transformed into the AH109 yeast strain for Y2H cDNA library screening. RNA isolated from *N. benthamiana* leaves was used for cDNA library construction. The cDNA library was generated with a Make Your Own "Mate & Plate" Library System (Clontech) following the manufacturer's instructions. Yeast colonies were picked and cultured in liquid medium lacking leucine and tryptophan for 16 h. Overnight culture was diluted to an OD₆₀₀ of 0.06, and 3 μ l was spotted on a selection plate containing histidine, leucine, tryptophan, and 5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside (X-Gal). Yeast colonies appearing on growth medium were picked and examined by PCR with the primer pair T7pro/FJJ2015-13. PCR products showing a single amplicon were sequenced. Sequences were used in a BLAST search against an *N. benthamiana* database in the Sol Genomics Network (<https://solgenomics.net/>). Protein domains were identified by the BLASTx algorithm and the Conserved Domain Database of the NCBI database (<http://www.ncbi.nlm.nih.gov/>). GO analysis was conducted using agriGO (<http://systemsbiology.cau.edu.cn/agriGOv2/index.php>). Plasmids were extracted from yeast cells, propagated in *Escherichia coli* DH5 α , and co-transformed with pGBKOMMP into yeast cells for verification.

4.4 | Construction of plasmid vectors used for transient silencing and overexpression

Partial or full-length genes were amplified with specific primers (Table S1) by RT-PCR and cloned into the Gateway entry vector pENTR/D-TOPO (Invitrogen). To construct transient silencing vectors, hpPPI and hpPDS fragments containing a hairpin loop were amplified by RT-PCR with the oligonucleotide pairs FJJ2017-125/FJJ2017-126 and FJJ2017-185/FJJ2017-186 (Table S1), respectively. The hpPPI and hpPDS fragments were cloned into pENTR/D-TOPO to generate pEN-hpPPI and pEN-hpPDS, respectively. The hpPPI fragment in pEN-hpPPI and the hpPDS fragment in pEN-hpPDS were transferred to the pK7GWIWG2(D) vector (Karimi et al., 2003) behind the CaMV 35S promoter using the LR Gateway cloning reaction (Invitrogen) to yield transient silencing vectors pK7-hpPPI and pK7-hpPDS, respectively. To construct transient overexpression vectors, full-length genes encoding MP^{OM}, NbFKPPIase, and GFP were amplified by PCR with the oligonucleotide pairs FJJ2018-13/FJJ2018-16, FJJ2017-167/FJJ2017-168, and FJJ2017-231/FJJ2017-232, respectively (Table S1). The amplified gene fragment encoding NbFKPPIase was fused with a c-myc tag at the 3'-terminus. The gene fragments encoding MP^{OM}, NbFKPPIase, and GFP were first cloned into the pENTR/D-TOPO vector to generate pEN-OMMP, pEN-PPIase, and pEN-GFP and then transferred to pK2GW7 (Karimi et al., 2003) to

generate transient overexpression vectors pK2-OMMP, pK2-PPlase, and pK2-GFP, respectively.

4.5 | BiFC and FRET analysis

In addition to pEN-PPlase, the MP^{OM} gene fragment was amplified by PCR with the primer pair FJJ2018-13/FJJ2018-17 lacking a termination codon and cloned into the pENTR/D-TOPO vector to produce pEN-MPnonstop. For BiFC analysis, NbFKPPlase and MP^{OM} coding sequences were transferred from the pENTR vector to pUBN-nYFP-Dest, pUBN-cYFP-Dest, pUBC-nYFP-Dest, and pUBC-cYFP-Dest destination vectors to generate pUBPPIInYFP, pUBPPIcYFP, pUB-nYFPPI, pUBcYFPPI, pUBMPnYFP, pUBMPcYFP, pUBnYFPMP, and pUBcYFPMP, which produced NbFKPPlase:nYFP, NbFKPPlase:cYFP, nYFP:NbFKPPlase, cYFP:NbFKPPlase, MP^{OM}:nYFP, MP^{OM}:cYFP, nYFP:MP^{OM}, and cYFP:MP^{OM} fusion proteins, respectively. These constructs were individually transformed into *Agrobacterium tumefaciens* C58. Agrobacteria containing these constructs were infiltrated into *N. benthamiana* leaves with combinations of pUBPPIInYFP/pUBMPcYFP, pUBPPIInYFP/pUBcYFPMP, pUBPPIcYFP/pUBMPnYFP, pUBPPIcYFP/pUBnYFPMP, pUBnYFPPI/pUBMPcYFP, pUBnYFPPI/pUBcYFPMP, pUBcYFPPI/pUBMPnYFP, or pUBcYFPPI/pUBnYFPMP.

For FRET analysis, the gene encoding NbFKPPlase was transferred from pEN-PPlase into the pK7WG2.0-C-CFP vector (Karimi et al., 2003) to generate the pKPPI-CFP construct, which could produce an NbFKPPlase:CFP fusion protein. The MP^{OM} gene fragment was transferred into pK7WG2.0-C-YFP (Karimi et al., 2003) to generate the pKMP-YFP construct, which could produce an MP^{OM}:YFP fusion protein. The constructs were individually transformed into *A. tumefaciens* C58. Agrobacteria containing the pKPPI-CFP and pKMP-YFP plasmids were individually infiltrated or co-infiltrated into *N. benthamiana* leaves for transient overexpression. For virus background analysis, agrobacteria containing fusion protein expression plasmids were co-infiltrated with infectious clones of ToLCNDV-OM into *N. benthamiana* leaves.

Three days after agro-infiltration, infiltrated leaves were collected and prepared in 0.5 × 0.5 mm² pieces for fluorescence observation. Visualization of fluorophores and FRET analysis were performed using an FV3000 confocal microscope (Olympus), and images were analysed using FV31S-SW software (Olympus).

4.6 | Agro-infiltration and begomovirus inoculation

Binary vector clones used for transient overexpression or silencing were transformed into *A. tumefaciens* C58 by electroporation. A single colony was picked and cultured overnight at 28°C in 4 ml lysogeny broth (LB) medium containing appropriate antibiotics. One millilitre of overnight culture was transferred into 10 ml LB medium containing appropriate antibiotics and 100 μM acetosyringone (AS) and further incubated at 28°C until reaching an OD₆₀₀ of 1.0. The culture was centrifuged at 5000 × g for 10 min, and the

Agrobacterium cells were resuspended in 20 ml infiltration medium (10 mM 2-(N-morpholino)ethanesulfonic acid, 10 mM MgCl₂, 100 μM AS) and incubated at room temperature for 2 h before infiltration. For transient overexpression and silencing, agrobacteria were directly infiltrated into *N. benthamiana* leaves. To analyse gene function, half of the leaf was infiltrated with overexpression or hairpin silencing construct of the target gene, and the other half was infiltrated with the control construct (GFP for overexpression and hpPDS for silencing control). For FRET analysis, agrobacteria containing pKCFP-PPI or pKMP-YFP were mixed at a 1:1 ratio, diluted 10-fold with infiltration buffer, and used for infiltration. Infectious clones of ToLCNDV-OM, ToLCNDV-CB, and TYLCTHV were constructed in our previous work (Lee et al., 2020). Agrobacteria carrying the infectious DNA-A or DNA-B constructs were co-injected in equal amounts into the leaves of *N. benthamiana*.

4.7 | Library preparation for transcriptome sequencing

N. benthamiana leaves after being infiltrated with agrobacteria containing the NbFKPPlase transient overexpression or silencing construct were collected for RNA extraction. A total amount of 1.5 μg RNA per sample was used for transcriptome analysis. Double-stranded cDNA was synthesized using the PrimeScript Double-Strand cDNA Synthesis Kit (Takara) following the manufacturer's recommendations. DNA was cleaned up with KAPA Pure Beads (KAPABiosystem; Roche) followed by end repair and A-tailing reaction with the KAPA HyperPrep Kit (KAPABiosystems). DNA was cleaned up for adaptor ligation and subjected to sequencing with a Direct cDNA Sequencing Kit (Oxford Nanopore Technology). A total amount of 100 pmol of cDNA was loaded on Nanopore R9.4 flow cells and sequenced using the MinION platform (Oxford Nanopore Technology).

4.8 | Base calling, alignment, and differential gene expression analysis

A Nanopore Albacore workflow was used for base calling direct cDNA sequencing data. Sequence read data were analysed with CLC Genomics Workbench v. 9.5.1. The draft genome sequence (Niben101_annotation.transcripts) of *N. benthamiana* was downloaded from the Sol Genomics Network FTP site as a reference for RNA sequencing analysis. DEG analysis was conducted by the proportion-based statistical analysis algorithm of CLC counting with transcripts per million bases (TPM). GO analysis of DEGs was performed by the agriGO web-based tool (<http://systemsbiology.cau.edu.cn/agriGOv2/index.php>).

ACKNOWLEDGEMENTS

We are grateful to Dr Kuang-Ren Chung for his critical review of this manuscript. This work was supported by grants from the Ministry

of Science and Technology (MOST 105-2313-B-005-019-MY3 and 108-2313-B-005-034 -MY3), Executive Yuan, Taiwan. This work was also financially supported (in part) by the Advanced Plant Biotechnology Center from The Featured Areas Research Center Program within the framework of the Higher Education Sprout Project by the Ministry of Education (MOE) in Taiwan. The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

Data are available on request from the authors. Accession numbers: ToLCNDV-OM (DNA-A, GU180095 and DNA-B, MK883714), ToLCNDV-CB (DNA-A, MK883715 and DNA-B, MK883716), TYLCTHV (DNA-A, GU723742 and DNA-B, GU723754), NbFKPase (Niben101Scf07001g01011.1), and Actin (Niben101Scf09133g02006.1).

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How to cite this article: Chang, H.-H., Lee, C.-H., Chang, C.-J. & Jan, F.-J. (2022) FKBP-type peptidyl-prolyl *cis-trans* isomerase interacts with the movement protein of tomato leaf curl New Delhi virus and impacts viral replication in *Nicotiana benthamiana*. *Molecular Plant Pathology*, 23, 561–575. <https://doi.org/10.1111/mpp.13181>