The C5 protein encoded by Ageratum leaf curl Sichuan virus is a virulence factor and contributes to the virus infection

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Abstract
Earlier reports have indicated that begomoviruses encode four proteins (AC1/C1, AC2/C2, AC3/C3, and AC4/C4 proteins) using complementary-sense DNA as the template. In recent years, several reports have shown that some begomoviruses also encode an AC5/C5 protein from the complementary DNA strand, and these AC5/C5 proteins play different roles in virus infections. Here, we provide evidence showing that Ageratum leaf curl Sichuan virus (ALCScV), a monopartite begomovirus, also encodes a C5 protein that is important for disease symptom formation and can affect viral replication. Infection of Nicotiana benthamiana plants with a potato virus X (PVX)-based vector carrying the ALCScV C5 gene resulted in more severe disease symptoms and higher virus accumulation levels. ALCScV C5 protein can be found in the cytoplasm and the nucleus. Furthermore, this protein is also a suppressor of posttranscriptional gene silencing. Mutational analysis showed that knockout of C5 gene expression significantly reduced ALCScV-induced disease symptoms and virus accumulation, while expression of the C5 gene using the PVX-based vector enhanced ALCScV accumulation in coinfected N. benthamiana plants.

KEYWORDS
ALCScV, C5 protein, pathogenicity, virus accumulation

1 | INTRODUCTION

Geminiviruses are single-stranded circular DNA viruses that infect many plant species and cause severe economic losses worldwide (Fauquet et al., 2003; Harrison, 1985; Navas-Castillo et al., 2011). Based on the current taxonomic criteria, geminiviruses are classified into nine genera: Becurtovirus, Begomovirus, Capulavirus, Curtovirus, Turncurtovirus, Eragrovirus, Grablovirus, Topocuvirus, and Mastrevirus (Varsani et al., 2014, 2017). Genus Begomovirus contains the largest number of virus species, which are transmitted by the whiteflies of the Bemisia tabaci complex and can infect many economically important dicotyledonous crop plants in subtropical and tropical countries (Mansoor et al., 2003; Zerbini et al., 2017). The Begomovirus genus can also be divided into two groups, Old World and New World, according to phylogenetic characteristics and genome organization, with monopartite and bipartite begomoviruses in both groups (Briddon et al., 2010; Fauquet et al., 2008; Fondong, 2013). Among them, the bipartite begomoviruses contain two genomic components (DNA-A and DNA-B), while monopartite begomoviruses contain a single genomic component that is equivalent to...
DNA-A of bipartite begomoviruses. Most reports have indicated that begomovirus DNA-A encodes six proteins: the coat protein (AV1/V1) and movement protein (AV2/V2) from the viral sense-strand DNA, and the replication-associated protein (AC1/C1), transcriptional-activator protein (AC2/C2), replication-enhancer protein (AC3/C3), and a multifunctional protein (AC4/C4) from the complementary-sense strand (Chen et al., 2019; Deom & Mills-Lujan, 2015; Harrison et al., 2002; Laufs et al., 1995; Padidam et al., 1996; Settlage et al., 2005; Sunter & Bisaro, 1991).

In recent years, several reports have also indicated that the DNA-A component of a few bipartite and monopartite begomoviruses encodes an AC5/C5 protein from the complementary-sense strand (Li et al., 2015). The known AC5/C5 proteins contain about 100 amino acid residues and are encoded from an open reading frame (ORF) located downstream of the C3 ORF. Most of the AC5/C5 ORF overlaps the 3’ half of the V1 ORF on the viral sense-strand. C5 proteins are known to be multifunctional. For example, the AC5 protein of mungbean yellow mosaic India virus (MYMIV) has 83 amino acid residues and plays key roles in MYMIV infection in plants and has RNA silencing suppression activity (Li et al., 2015). Tomato chlorotic mottle virus (ToCMoV) AC5 protein has 250 amino acid residues and is not essential for virus infection, based on mutagenesis analysis (Fontenelle et al., 2007). Watermelon chlorotic stunt virus (WmCSV) AC5 protein has 255 amino acid residues and is not required for WmCSV infectivity in plants (Kheyr-Pour et al., 2000). Ageratum leaf curl Sichuan virus (ALCScV) was first identified in 2018 in Ageratum conyzoides in Sichuan Province, China. ALCScV is a monopartite begomovirus with no known alphasatellite or betasatellite (Li et al., 2018). To date, the C4 protein of ALCScV has been shown to function in disease symptom formation and virus accumulation (Li et al., 2020). In this study, we demonstrated that ALCScV encodes a C5 protein. Subsequently, the pathogenicity of ALCScV C5 protein was analysed via expression with the potato virus X (PVX) and enhances the pathogenicity of PVX in N. benthamiana plants.

2 | RESULTS

2.1 | Sequence analysis of ALCScV-encoded C5 protein

The ALCScV sequence was analysed using the Open Reading Frame Finder software at the NCBI (https://www.ncbi.nlm.nih.gov/orffinder/) and it was found that ALCScV can encode a C5 protein with 115 amino acid residues. The ALCScV C5 ORF is on the viral complementary-sense strand DNA, nucleotide positions 781 to 1,125, and overlaps the V1 ORF on the viral sense-strand (Figure 1a). To further investigate this protein, we aligned C5 amino acid sequences from 12 begomoviruses using the ClustalV program in the DNASTar v. 6.0 software (DNASTar Inc.) and generated a phylogenetic tree for these C5 proteins. The result indicated that the ALCScV C5 protein is most closely related to the MYMIV C5 protein (Figure 1b).

2.2 | Detection of the C5 protein during viral infection

To confirm the expression of C5 in ALCScV-infected plants, total RNA was extracted from the leaves harvested from ALCScV-inoculated or mock-inoculated plants using TRizol reagent. The C5-specific primers were used to reverse transcribe RNA into cDNA. PCR was then performed using a pair of C5 gene-specific primers, C5-F/C5-R (Table S1). The result confirmed that C5 RNA transcripts had accumulated in the samples harvested from the ALCScV-infected plants but not from the mock-inoculated control plants (Figure 2a).

Like other gene expressions, the expression of C5 gene requires a promoter. To confirm the presence of a C5 gene promoter, a fragment of 500 nucleotides (nt) located upstream of the C5 ORF was PCR-amplified and used to replace the 35S promoter in the expression vector pCHF3-GFP to produce vector pCHF3-500-GFP. After transformation of these two expression vectors into Agrobacterium tumefaciens C58C1 cells and agroinfiltration into N. benthamiana leaves, green fluorescent protein (GFP) fluorescence was observed in the pCHF3-500-GFP-infiltrated leaves using laser-scanning confocal microscopy (LSCM) at 2 days postagroinfiltration (dpi) (Figure S1), although the strength of green fluorescence in the pCHF3-500-GFP-infiltrated leaves was weaker than that in the pCHF3-GFP-infiltrated leaves. Western blot analysis of GFP accumulation in the infiltrated leaf samples confirmed that this 500 nt fragment has promoter activity (Figure 2b).

2.3 | C5 is a virulence factor and enhances the pathogenicity of PVX in N. benthamiana

To investigate the function of C5 protein during virus infection in plants, we constructed a PVX expression vector carrying the ALCScV C5 gene (PVX-C5) and inoculated it to N. benthamiana plants. By 7 dpi, the PVX-C5-inoculated plants developed severe chlorosis or yellow mosaic in leaves, while the PVX-inoculated control plants showed only mild mosaic symptoms in leaves (Figure 3a). To further confirm the effect of C5 on PVX accumulation in the infected plants, we analysed the systemic leaf tissues harvested from the PVX- or the PVX-C5-inoculated plants using quantitative reverse transcription PCR (RT-qPCR). The result showed that PVX RNA accumulated more in the PVX-C5-inoculated plants compared with that in the PVX-inoculated plants (Figure 3b). Consequently, we conclude...
that ALCScV C5 protein is a determinant of PVX pathogenicity in *N. benthamiana* plants.

### 2.4 | Subcellular localization

Correct subcellular localization of proteins is essential for protein function (Luo et al., 2014; Mei et al., 2018a). In this study, we transiently expressed GFP and C5-GFP, respectively, in *N. benthamiana* leaves to determine the subcellular localization pattern of the C5 protein. By 2 days postinoculation (dpi), the C5-GFP fusion protein was observed in the cytoplasm and nucleus under an LSCM (Figure 4a). To further confirm this finding, we analysed assayed leaf tissues by western blot assays. The result showed that a protein band with a higher molecular mass, a feature indicative of the C5-GFP fusion protein, was detected in the leaf tissues agroinfiltrated with pCHF3-C5-GFP (Figure 4b). Further colocalization results showed that the V1-RFP fusion protein and C5-GFP fusion protein were observed in the cytoplasm and the nucleus under an LSCM (Figure 4c).
2.5 | C5 protein is a suppressor of RNA silencing

To identify whether the C5 protein is a suppressor of RNA silencing we co-inoculated GFP-transgenic *N. benthamiana* 16c plants with pCHF3 + 35S:GFP, pCHF3-C5 + 35S:GFP, and pCHF3-p19 + 35S:GFP (a vector expressing the tomato bushy stunt virus P19 gene as a positive control). At 5 dpai, the leaf expressing pCHF3-p19 + 35S:GFP showed strong green fluorescence under UV light, and the leaves inoculated with pCHF3-C5 + 35S:GFP showed mild green fluorescence. In contrast, no obvious green fluorescence was observed with *N. benthamiana* 16c plants inoculated with pCHF3 + 35S:GFP (Figure 5a). Then, the relative GFP protein accumulation levels were analysed by western blot, and the results showed that the accumulation of GFP proteins was significantly higher in the infiltrated leaves with pCHF3-p19 + 35S:GFP or pCHF3-C5 + 35S:GFP than in those inoculated with pCHF3 + 35S:GFP (Figure 5b). These results suggest that C5 can suppress PTGS in the plants.

2.6 | C5 contributes to ALCScV infection in *N. benthamiana*

To investigate role of the C5 protein in ALCScV infection in *N. benthamiana*, we constructed a C5 gene knockout ALCScV clone (referred to as ALCScV-mC5) by mutagenizing the translation initiation codon of C5. The wildtype ALCScV and the ALCScV-mC5 mutant were inoculated to *N. benthamiana* plants via agroinfiltration using needless syringes. By 14 dpai, the ALCScV-inoculated *N. benthamiana* control plants showed leaf curling and plant stunting symptoms. In contrast, no clear disease symptoms were observed on the wildtype *N. benthamiana* plants inoculated with ALCScV-mC5 (Figure S2). By 21 dpai, the ALCScV-inoculated *N. benthamiana* plants showed stronger leaf curling and plant stunting symptoms, while the ALCScV-mC5-inoculated *N. benthamiana* plants showed only mild leaf-curling symptoms (Figure 6a). Meanwhile, the wildtype ALCScV and the ALCScV-mC5 mutant were also inoculated to *Helianthus annuus* plants. Similarly, inoculation with wildtype ALCScV induced more severe symptoms in *H. annuus* plants than inoculation with ALCScV-mC5 mutant (Figure S3). Analysis of virus accumulation in the infected *N. benthamiana* plants at 7, 14, 21, and 28 dpai through quantitative PCR (qPCR) showed that the ALCScV-mC5-infected plants had accumulated significantly less viral DNA compared with the ALCScV-infected plants (Figure 6b), indicating that the C5 protein is important for ALCScV infection in *N. benthamiana*.

2.7 | Overexpression of ALCScV C5 protein enhanced ALCScV accumulation in *N. benthamiana*

In the next experiment, we co-inoculated *N. benthamiana* plants with ALCScV and PVX (ALCScV + PVX), ALCScV and PVX-C5 (ALCScV + PVX-C5), ALCScV-mC5 and PVX (ALCScV-mC5 + PVX), and ALCScV-mC5 and PVX-C5 (ALCScV-mC5 + PVX-C5) through agroinfiltration. By 14 dpai, the ALCScV-mC5 + PVX-C5-inoculated plants...
**FIGURE 4** Subcellular localization of ALCScV C5 protein in *Nicotiana benthamiana* leaf cells. (a) Green fluorescent protein (GFP) and C5-GFP fusion were transiently expressed in *N. benthamiana* leaf cells through agroinfiltration. Accumulation of C5-GFP in the nucleus is indicated by white arrows. Images are captured using laser-scanning confocal microscopy at 2 days postagroinfiltration (dpai). (b) Western blot analysis of GFP and C5-GFP fusion protein accumulation in the leaves at 2 dpai. The blot was probed with an anti-GFP antibody. Coomassie brilliant blue G-250 stained gel was used to show sample loadings. (c) Colocalization of V1-RFP fusion protein and C5-GFP fusion protein in *N. benthamiana* leaf cells. The white arrow represents the nucleus.

*N. benthamiana* plants showed leaf yellowing and curling symptoms similar to those shown by the ALCScV + PVX-C5- or ALCScV + PVX-inoculated plants, but stronger than those shown by the ALCScV-mC5 + PVX-inoculated plants (Figure 7a). Analysis of these plants through qPCR at 14 dpai showed that expression of C5 protein using the PVX vector significantly increased viral DNA accumulation, especially the ALCScV DNA (Figure 7b), indicating that the C5 protein is important for disease symptom formation and virus accumulation.

**3 | DISCUSSION**

Earlier reports had indicated that begomoviruses encode two proteins from the viral-sense DNA-A and four proteins from the complementary-sense DNA-A. In recent years, several reports have shown that the DNA-A component of some begomoviruses, including MYMIV, tomato leaf deformation virus (ToLDeV), ToCMoV, and WmCSV, also encode an AC5/C5 protein (Fontenelle et al., 2007; Kheyr-Pour et al., 2000; Li et al., 2015; Melgarejo et al., 2013). ALCScV causes typical begomovirus-induced disease symptoms in *H. annuus* and *N. benthamiana* plants (Li et al., 2020b). In this study, we have determined that like some other begomoviruses, ALCScV also encodes a C5 protein with 115 amino acid residues from a region (nucleotides 781 to 1,125) on the complementary-sense DNA. The C5 ORF overlaps the 3′ half of the V1 ORF on the viral-sense DNA.

Many studies have indicated that begomoviruses encode different proteins associated with disease symptom formation in plants. For example, the V2 protein of beet curly top virus (BCTV) can induce severe systemic necrosis in *N. benthamiana* plants, the C1 protein of apple geminivirus (AGV) can induce severe plant dwarfing symptoms, and the C4 protein of Malvastrum yellow vein virus (MaYVV) can induce *N. benthamiana* leaf upward rolling symptoms (Jing et al., 2019; Luna et al., 2020; Zhan et al., 2018). It is noteworthy that the AC5/C5 proteins of begomoviruses have different functions in virus infections. For example, the MYMIV AC5 protein is a virulence factor with a specific role in MYMIV pathogenicity (Li et al., 2015). Nevertheless, the AC5 protein of other bipartite begomovirus WmCSV and ToCMoV are not required for virus infection and symptom formation (Fontenelle et al., 2007; Kheyr-Pour et al., 2000). Interestingly, although the C5 gene encoded by two isolates of ToLDeV does not affect virus infection, a C5 knockout mutant causes milder disease symptoms in *N. benthamiana* plants.
than the wildtype virus. (Melgarejo et al., 2013). Here, we provide
evidence to show that expression of ALCScV C5 protein using a
PVX-based vector can cause severe chlorosis and mild leaf mal-
formation in *N. benthamiana* plants. This C5 protein can also pro-
mote PVX viral RNA accumulation in plants. Mutational analysis
demonstrated that the mutant ALCScV defective in C5 expression
failed to cause clear disease symptoms in the infected plants and
accumulated much less viral DNA. In contrast, coinfection of *N. ben-
thamiana* plants with ALCScV + PVX-C5 or ALCScV-mC5 + PVX-C5
significantly increased viral DNA accumulation compared with the

**FIGURE 5** Suppression of local posttranscriptional gene silencing in *Nicotiana benthamiana* 16c transgenic plants by ALCScV C5 protein. (a) pCHF3-C5 + pCHF3-GFP or pCHF3-p19 + pCHF3-GFP were coinfiltrated into *N. benthamiana* 16c plants. Leaves expressing pCHF3-p19 + pCHF3-GFP and pCHF3-C5 + pCHF3-GFP showed green fluorescence under UV light at 5 days postagroinfiltration. (b) Western blot analysis of GFP protein accumulation in *N. benthamiana* leaves inoculated with pCHF3, pCHF3-C5 + pCHF3-GFP, or pCHF3-p19 + pCHF3-GFP. The blot was probed with an anti-GFP antibody. Coomassie brilliant blue G-250 stained gel was used to show sample loadings

**FIGURE 6** Infection of ALCScV and ALCScV-mC5 (knockout mutant) in *Nicotiana benthamiana* plants. (a) By 21 days postagroinfiltration (dpi) the ALCScV-mC5-inoculated plants showed mild downward leaf-curving symptoms, while the ALCScV-inoculated plants showed clear leaf-curving and plant-stunting symptoms. Representative assayed *N. benthamiana* plants were photographed at 21 dpi. (b) Detection of viral DNA accumulation in the ALCScV-inoculated and the ALCScV-mC5-inoculated *N. benthamiana* plants through quantitative PCR at various times after inoculation. Three plants were analysed for each treatment and three technical replicates were used for each biological sample using quantitative PCR. **p < .01, Student’s t test. The experiment was repeated three times with similar results.**
plants coinfected with ALCScV + PVX, ALCScV + PVX-C5, ALCScV-mC5 + PVX, or ALCScV-mC5 + PVX-C5 were photographed at 14 days postagroinfiltration. (b) The assayed plants were analysed for ALCScV DNA accumulation through quantitative PCR using ALCScV-specific primers. Three plants were analysed for each treatment and three technical replicates were used for each biological sample during quantitative PCR. Different lower case letters indicate statistical significance as determined using Student’s t test, p ≤ .05. The experiment was repeated three times with similar results.

**FIGURE 7** ALCScV C5 protein affects disease symptom formation and viral DNA accumulation in *Nicotiana benthamiana*. (a) Representative *N. benthamiana* plants coinfected with ALCScV + PVX, ALCScV + PVX-C5, ALCScV-mC5 + PVX, or ALCScV-mC5 + PVX-C5 were photographed at 14 days postagroinfiltration. (b) The assayed plants were analysed for ALCScV DNA accumulation through quantitative PCR using ALCScV-specific primers. Three plants were analysed for each treatment and three technical replicates were used for each biological sample during quantitative PCR. Different lower case letters indicate statistical significance as determined using Student’s t test, p ≤ .05. The experiment was repeated three times with similar results.
betalatellites is a suppressor of PTGS. ALCSvC is a monopartite begomovirus with no known alphasatellite or betasatellite. Therefore, we speculate that ALCSvC C5 protein may replace part of the function of pC1 protein, for example the function of C5 protein as a suppressor of RNA silencing is a key strategy to break host defences.

ALCSvC can successfully establish systemic infection and induce typical viral symptoms in A. conyzoides, H. annuus, and N. benthamiana plants. Our previous research demonstrated that the C4 protein of ALCSvC is closely related to disease symptom formation and virus accumulation (Li et al., 2020b). In this study, we found that ALCSvC C5 protein also contributes to ALCSvC infection in N. benthamiana; however, the pathogenic function of the C5 protein is weaker than that of C4 protein. Further analysis of ALCSvC C4 and C5 proteins found that they also have many common features, for example they can enhance the pathogenicity of PVX in N. benthamiana, and they are both located in the cytoplasm and nucleus. Therefore, we speculate that C5 is also a multifunctional protein similar to C4. Whether the two proteins work together to promote the pathogenicity of ALCSvC remains to be studied in the future.

In summary, we demonstrated that ALCSvC encodes a C5 protein, which is a virulence factor and enhances the pathogenicity of PVX in N. benthamiana. The C5 protein is located in the cytoplasm and nucleus and is also a suppressor of PTGS. The ALCSvC C5 protein is important for disease symptom formation and can also affect ALCSvC replication in the infected plants.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plant materials and growth conditions

Wildtype and N. benthamiana 16c plants were grown inside a growth chamber set at 26 °C and 16 hr/8 hr (light/dark) photoperiod.

4.2 | Sequence analysis

ALCSvC ORF C5 was predicted and identified using Open Reading Frame Finder software at the NCBI (https://www.ncbi.nlm.nih.gov/orffinder/). Multiple sequence alignment of other C5 protein sequences was performed using the ClustalV alignment method by DNASTar software v. 6.0 (DNASTar Inc.). Phylogenetic analysis based on the amino acid sequences of C5 proteins from 12 begomoviruses was performed in MEGA 5.0 by the neighbour-joining method with 1,000 bootstrap replications. GenBank accession numbers are shown in the trees, and the names of the viruses are as follows: AEV, Ageratum enation virus (AWK29725); AYVCNv, Ageratum yellow vein China virus (QHZ10081); AYYVv, Ageratum yellow vein virus (QIH55855); CLCMv, cotton leaf curl Multan virus (ARI21940); CLCV, cotton leaf curl virus (AOW71925); MaYVV, Malvastrum yellow vein virus (QI32361); MYMV, Mesta yellow vein mosaic virus (AHX25706); MYMV, mungbean yellow mosaic India virus (AM950268); OELCV, okra enation leaf curl virus (QCO31519); SLCV, squash leaf curl China virus (QIH55808); TbcSV, tobacco curly shoot virus (QIH12595); TLCNDV, tomato leaf curl New Delhi virus (CUR44438); ToCMoV, tomato chlorotic mottle virus (AY090557); ToloDV, tomato leaf deformation virus (JX501504); WmCSV, watermelon chlorotic stunt virus (AJ245650).

4.3 | Plasmid construction

To examine the strength of the C5 promoter, the 500 nt fragments upstream of the C5 translation initiation site were amplified with the corresponding primer pairs (Table S1) using the amplified fragment to replace the 35S promoter in the binary expression vector pCHF3-GFP. The recombinant vector was named 500-GFP.

To construct the PVX-C5 vector, the full-length sequences of C5 (345 bp) were PCR-amplified using primers PVX-C5-F and PVX-C5-R (Table S1). Then, the PCR products were digested with the restriction enzymes ClaI and SalI, and inserted into the ClaI/SalI sites of the pGR106 vector to generate PVX-C5.

To construct binary plasmids transiently expressing C5-GFP fusion proteins for subcellular localization assays the coding sequences of C5 (with the termination codon TAA deleted) were amplified with the corresponding primer pairs pCHF3-C5-GFP-F/pCHF3-C5-GFP-R (Table S1), digested with BgIII and HindIII, then inserted into the binary vector pCHF3 to generate the recombinant vectors pCHF3-C5-GFP. The full-length sequence of V1 (774 bp, with the termination codon TAA deleted) was amplified using specific primers (Table S1) and then inserted into the pGD vector to produce pGD-V1-RFP.

For PTGS suppression assay, the full-length sequence of C5 (345 bp) was amplified using primers pCHF3-C5-F/pCHF3-C5-R (Table S1) and then inserted into the pCHF3 vector to produce pCHF3-C5.

To construct an infectious clone of an ALCSvC mutant that does not encode the C5 protein and does not affect the normal coding of other proteins, the full-length ALCSvC sequence was amplified using the primers SC782-InFu-F1 and SC782-InFu-R1, and the PCR fragment was cloned into the pGEM-T Easy vector (Promega). Then, we used the 2x TransStart FastPfu PCR SuperMix Kit (Transgen Biotech) and a pair of primers SC782-mC5-F1/SC782-mC5-R1 to mutate the potential translation start codons (ATG) in the C5 gene to ACG codons. The infectious clone of ALCSvC is described in Li et al. (2020b). After the potential translation start codon was successfully mutated, two pairs of primers, SC782-InFu-F1/SC782-InFu-R1 and SC782-InFu-F2/SC782-InFu-R2, were used to amplify the full-length genome (1.0A) and c.0.9-mer fragment (0.9A) of ALCSvC, respectively. Through a seamless cloning method, the 1.0A and 0.9A were inserted into the pBinPLUS vector to produce pBinPLUS-1.9AmC5.

4.4 | Agroinfiltration assays

For inoculation of plants, the recombinant 500-GFP vector was transformed into A. tumefaciens C58C1. The PVX-based expression
vectors and pBinPLUS vector were transformed into A. tumefaciens GV3101psa. A. tumefaciens C58C1 and GV3101psa were grown at 28 °C for 2 days on Luria Bertani (LB) solid medium supplemented with spectinomycin (50 μg/ml) and rifampicin (20 μg/ml), and rifampicin (20 μg/ml) and kanamycin (50 μg/ml), respectively. Then, the A. tumefaciens was transferred to LB liquid medium containing the corresponding antibiotics and cultivated overnight in a 28 °C shaker. The cultures were centrifuged and resuspended in infiltration buffer (10 mM MES, 200 μM acetylserine, 10 mM MgCl2) to OD600 = 1.0, then infiltrated into leaves of 4–6-leaf stage N. benthamiana plants using a sterile syringe.

4.5 | Total RNA extraction and qPCR analysis

Total RNA was extracted from leaf samples using TRIzol reagent. Total RNA quantity and purity were assayed by spectrophotometer. Then, the total RNA was reverse transcribed to complementary DNA (cDNA) using the PrimeScript RT reagent Kit. qPCR was conducted using NovoStart SYBR qPCR Super Mix Plus (Novoprotein) on CFX 96 Real-Time System (Bio-Rad). NbActin gene was selected as an internal control for the assays. All the gene expression data were performed with three biological replicates and three technical replicates, and the relative gene expression levels calculated using the 2−ΔΔCT method for analysis.

4.6 | DNA extraction and viral DNA accumulation analysis

To determine the viral DNA accumulation levels in the infected tissue samples. The total DNA was extracted from the newly emerged leaves using the CTAB method. Before qPCR, the quantity and purity of total DNA were measured by spectrophotometer and concentration were standardized to 100 ng/μl. The relative accumulation levels of viral DNA were measured using the qPCR assays as described by Li et al. (2020b).

4.7 | Protein extraction and western blot analysis

Total protein was extracted from inoculated leaves using protein extraction buffer. The total proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and the protein samples were transferred to polyvinylidene difluoride (PVDF) membrane. The anti-GFP monoclonal antibody was used at a 1:2,000 dilution and incubated with membranes for 2 hr. Subsequently, the membranes were transferred to 5% skim milk solution containing horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody. Finally, the blot was incubated in Super ECL plus western blotting kit (US Everbright Inc.) for the detection of chemiluminescence.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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