Hue University Journal of Science: Agriculture and Rural Development
Vol. 135, No. 3D, 21–30, 2026

pISSN 2588-1191
eISSN 2615-9708

Effect of *ac4*-null mutations on the infectivity of pepper yellow leaf curl Thailand virus in *nicotiana benthamiana*

Ho Ngoc Han[1]*, Ho Thi Hoang Nhi[1], Huynh Thi Thanh Tuyen[2], Truong Thi Hong Hai[1]

[1] Institute of Biotechnology, Hue University, Nguyen Dinh Tu St. Hue City, Vietnam
[2] University of Science and Technology, The University of Da Nang,
54 Nguyen Luong Bang St., Da Nang City, Viet Nam

* Correspondence to: Ho Ngoc Han < hongochan@hueuni.edu.vn>
(Received: 13 Jan, 2026; Accepted: 17 March, 2026)

Abstract. Begomoviruses are among the most devastating plant viruses. They have compact genomes that encode six to eight proteins important for viral replication and spread. Among these proteins, AC4 (C4 in monopartite begomoviruses) is a multifunctional protein with roles in host immunity suppression and disease symptom expression. Here, we investigated the role of *AC4* in the infectivity of pepper yellow leaf curl Thailand virus, a begomovirus recently reported in central Vietnam. First, three *AC4*-null mutants were created from the infectious clones of DNA-A of PepYLCThV using QuikChange site-directed mutagenesis. The mutants were confirmed with PCR and DNA sequencing. The wild-type and mutants infectious clones were used to infect *Nicotiana benthamiana* via agroinoculation. Symptom expression was monitored for up to 28 days post inoculation. Disease onset started at five to six days post inoculation. Inoculation with *AC4*-null mutant infectious clones, similar to wild-type *AC4* infectious clones, resulted in severe leaf distortion, mosaic, white patches on leaves and stunted growth in 100% of inoculated plants. Furthermore, the presence of DNA-A and DNA-B were confirmed in infected plants by PCR. These results suggested *AC4* plays an insignificant role in the infectivity of PepYLCThV in the model plant *N. benthamiana*.

Keywords: agroinoculation, begomovirus, pepper yellow leaf curl Thailand virus

1 Introduction

Begomovirus is a genus of plant viruses in the family Geminiviridae [1]. These viruses are primarily transmitted by the whitefly (*Bemisia tabaci*) and cause significant damage to crops worldwide. They infect a wide range of dicotyledonous plants and are responsible for several economically important diseases, such as tomato yellow leaf curl disease, cassava mosaic disease and pepper yellow leaf curl disease [2-4]. Symptoms typically include leaf curling, yellowing, stunted growth, and reduced yield. Management strategies for begomovirus infections include breeding resistant crop varieties, controlling whitefly populations, using clean planting materials, and applying integrated pest management practices [2,3, 5-7].

Begomoviruses have compact genomes that include a circular, single-stranded DNA (monopartite begomoviruses) or two circular, single-stranded DNA (bipartite begomoviruses). Their genomes encode six to eight proteins that are important for viral replication and movement [8].

Among these genes, *AC4* (*C4* in monopartite begomoviruses) encodes a protein of more than 80 amino acid residues, with low level of sequence conservation, corresponding to functional diversity across begomovirus species [9]. Possibly due to this diversity, studies on the role of *AC4* in virus replication and disease expression have yielded conflicting results across different species. For example, *AC4* in tomato golden mosaic virus is not required for disease expression in tomato [10]. In contrast, the *AC4* genes in African cassava mosaic virus, mung bean yellow mosaic virus, East African cassava mosaic Cameroon virus and tomato leaf curl Hsinchu virus are important for spread and disease expression, although not required for virus replication [11-14]. Furthermore, protein AC4 was found to interact with various host proteins, including receptor-like kinases (RLKs) like CLV1, Shaggy-like kinases, and AGO4, SGS3/RDR6, influencing cell division, meristem development and viral DNA methylation [15,16]. In Sri Lankan cassava mosaic virus, AC4 acts as a suppressor of post-transcriptional gene silencing (PTGS), a plant defense mechanism that targets viral RNA for degradation [17]. AC4 directly binds UPF1, a translational suppressor, and targets UPF1 for degradation through the autophagy and ubiquitin–proteasome pathways [18]. These represent multiple pathways via which AC4 helps begomoviruses to evade the host's immune response and establish a persistent infection.

To further understand the role of AC4 in other begomoviruses, we turned towards pepper yellow leaf curl Thailand virus (PepYLCThV), a bipartite begomovirus that infects chili pepper (*Capsicum annuum*) [4]. In PepYLCThV, the *AC4* gene encodes a 11.2 kDa protein, whose function has not been characterized. To determine whether *AC4* plays a role in PepYLCThV infectivity, we created three *AC4*-null infectious clones, based on the sequence of PepYLCThV isolate QNam01 (collected in Quang Nam, central Vietnam) [19]. Three *AC4*-null infectious clones were created by QuikChange site-directed mutagenesis to introduce a termination codon into the *AC4* coding sequence without changing the amino acid sequence of the overlapping *AC1* gene. These *AC4*-null infectious clones were used to infect *Nicotiana benthamiana*, the symptom expression and infectivity rate were monitored for up to 28 days post inoculation.

2 Methodology

2.1 Construction of PepYLCThV *AC4*-null infectious clones

PepYLCThV samples were collected and the construction of PepYLCThV DNA-A and DNA-B infectious clones was reported in our previous work [19]. Briefly, the entire DNA-A and DNA-B of PepYLCThV were amplified and cloned between the two *Sap*I sites on pLX-AS [20], using Gibson assembly [21]. The resulting infectious clones for DNA-A and DNA-B were called pLX-Pep-A and pLX-Pep-B, respectively (Figure 1).

AC4-null mutants were created with a non-sense mutation in the open reading frame (ORF) of the gene *AC4*. Since *AC4* ORF overlaps *AC1* ORF, the gene encodes replication-associated protein, a mutation in *AC4* might result in a mutation in *AC1* as well. To create *AC4*-null mutants without changing the *AC1* amino acid sequence, we chose to insert a stop codon right after amino acid position F8, T25 and M47 (in three plasmids pLX-Pep-A(F8*), pLX-Pep-A(T25*) and pLX-Pep-A(M47*)). These plasmids were created from pLX-Pep-A, using the QuikChange site-directed mutagenesis as previously described [22]. Briefly, the plasmid template (pLX-Pep-A) was amplified with primers containing the intended mutation. PCRs (20 μL) contained pLX-Pep-A (20 ng), Phusion™ high-fidelity polymerase and 0.5 μM forward and reverse primers (Table 1). PCR thermocycling involved an initial denaturation of

pISSN 2588-1191
eISSN 2615-9708

98°C for 30 seconds, followed by 34 cycles of amplification (98°C - 5 seconds, 52°C - 10 seconds

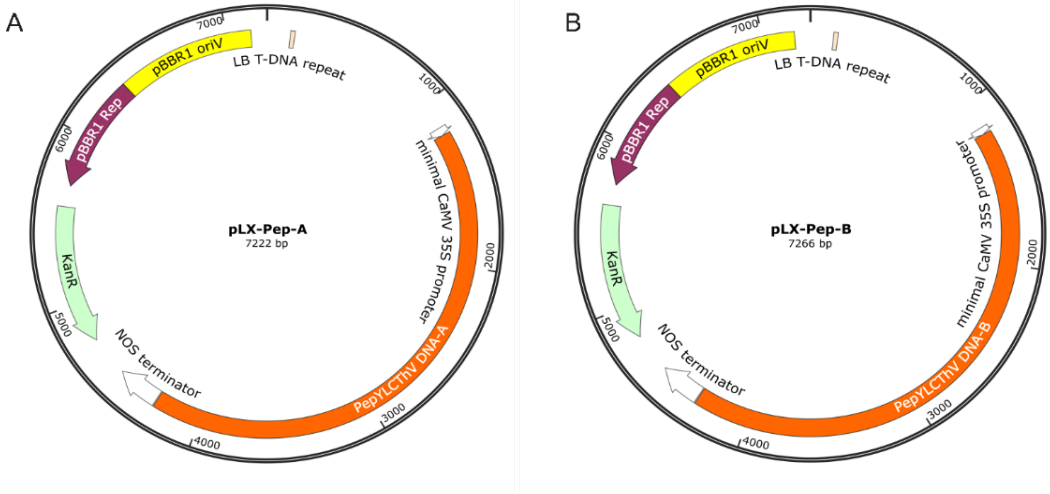

Figure 1. Maps demonstrate essential elements of the infectious clones pLX-Pep-A (A) and pLX-Pep-B (B).

and 72°C - 120 seconds) and a final extension (72°C for 5 minutes). PCR products were treated with *Dpn*I (Invitrogen, USA) at 37°C for 1 hour, followed by enzymatic denaturation (80°C for 20 minutes). The reaction mix was transformed into chemically competent *E. coli* DH5α, selected on LB plates containing kanamycin (50 µg/mL) and screened with colony PCR (Table 1). PCR products were resolved on 1.5% agarose gel in 0.5x TBE buffer, stained with ethidium bromide and visualized with a Gel documentation system (VILBER). Resulting plasmids were extracted from colonies yielding PCR product of the right size. The *AC4* sequences were verified with Sanger DNA sequencing (Apical Scientific, Malaysia).

Table 1. Forward and reverse primers used in the QuikChange site-directed mutagenesis to create *AC4*-null DNA-A infectious clones. The screening primers were used to screen *E. coli* colonies containing the mutants.

Mutagenesis primer		**Sequence (5′-3′)**
AC4(F8*)	AC4-F8-F	CATGTTCT**A**ATCCAATTCGAGGGTAAATTC
	AC4-F8-R	GAATTGGA**T**TAGAACATGCAAGTGAGGAGT
AC4(T25*)	AC4-T25-F	TCTTCGACCT**A**ACATCCACAACCAGGTCA
	AC4-T25-R	TGTGGATGT**T**AGGTCGAAGAATCTTTGATTC
AC4(M47*)	AC4-M47-F	CTTCAGATGT**A**AAGGCATACATGGAGAAAG
	AC4-M47-R	GTATGCCTT**T**ACATCTGAAGAGCTCTTAG
Screening primer		**Sequence (5′-3′)**
AC4(F8*)	LX-Pep-A-F2	TAGAGGGGATTGTTGACCG

(1053 bp)	AC4-F8-conf	CTCCTCACTTGCATGTTCTA
AC4(T25*)	LX-Pep-A-F2	TAGAGGGGATTGTTGACCG
(1002 bp)	AC4-T25-conf	ATCAAAGATTCTTCGACCTA
AC4(M47*)	LX-Pep-A-F2	TAGAGGGGATTGTTGACCG
(936 bp)	AC4-M47-conf	CTAAGAGCTCTTCAGATGTA

2.2 Agroinoculation of *N. benthamiana*

Agroinoculation was performed as previously described [23, 24]. Briefly, pLX-Pep-A, pLX-Pep-A(F8*), pLX-Pep-A(T25*), pLX-Pep-A(M47*) and pLX-Pep-B were separately transformed into *Agrobacterium tumefaciens* EHA105 by electroporation, selected on LB plates containing rifampicin (25 μg/mL) and kanamycin (50 μg/mL). Young *N. benthamiana* plants (six-leaf stage) were used for agroinoculation with infectious clones. The EHA105 bacterial pellets containing the DNA-B infectious clone were picked with sterile toothpicks and rubbed on the stem of *N. benthamiana* (near the shoot), followed by rubbing with the bacterial pellets containing one of the DNA-A infectious clones. For mock treatment, only the DNA-B infectious clone was rubbed on the stem as DNA-B alone was not capable of causing disease in *N. benthamiana* [10,24,25]. Wounds were created by stabbing the rubbed sites five times with a sterile insulin needle. The plants were maintained at 25 °C with a 16h light/8h dark cycle, and symptom expression was monitored for 28 days post inoculation. The experiment included three repeats, repeat 1 involved two biological replicates per treatment and repeat 2 and 3 involved three biological replicates per treatment.

Twenty-eight days post inoculation, direct PCR was performed to confirm the presence of DNA-A and DNA-B in infected plants. DNA templates were prepared by scraping the underside of *N. benthamiana* leaves and dipped in 20 μL of TE buffer (pH 8.0). PCR (20 μL) contained Phire Hot Start II DNA Polymerase, 0.5 μL of the DNA template, forward and reverse primers (0.25 μM, Table 2). PCR thermocycling involved an initial denaturation of 98 °C for 5 mins, 42 cycles of amplification (98°C for 5 sec, 61°C for 5 sec, 72 °C for 20 sec) and a final extension of 72 °C for 1 min. PCR products were resolved on 1.5% agarose gel in 0.5x TBE buffer, stained in ethidium bromide and visualized in a gel documentation system (VILBER).

Table 2. Forward and reverse primers used in PCR for the confirmation of PepYLCThV DNA-A and DNA-B in infected *N. benthamiana*.

	Primer	**Sequence (5′-3′)**
DNA-A	Pep-A-F1	GAAGCGTCCCGCAGATATAG
(1108 bp)	Pep-A-R1	GACGGTCAACAATCCCCT
DNA-B	Pep-B-F1	GCATGMGTGAAGCAGATG
(924 bp)	Pep-B-R1	GTACAGGGTGGAAGACACTA

pISSN 2588-1191
eISSN 2615-9708

3 Results and discussion

3.1 Construction of *AC4*-null infectious clones

To create *AC4*-null mutants, we introduced non-sense mutation right after amino acid F8, T25 or M47 on *AC4* from the QNam01 isolate (Figure 2A). These mutations were selected because the termination codon can be introduced in *AC4* without changing the amino acid sequence of the overlapping *AC1* gene. The mutation was created on pLX-Pep-A (sequence in Table S1) using the QuikChange site-directed mutagenesis. The mutant plasmids were screened using PCR primers that annealed to the mutant but not the wild-type sequence. We successfully detected mutant DNA with stop codon after amino acid F8, T25 and M47 (Figure 2B-C). The rates of colonies containing mutant plasmids were high, varying from 40% (pLX-Pep-A(T25*)) to 100% (pLX-Pep-A(M47*)). The resulting plasmids were purified and Sanger sequencing confirmed the *AC4* sequences contained the desired mutations (Figure 2D-F).

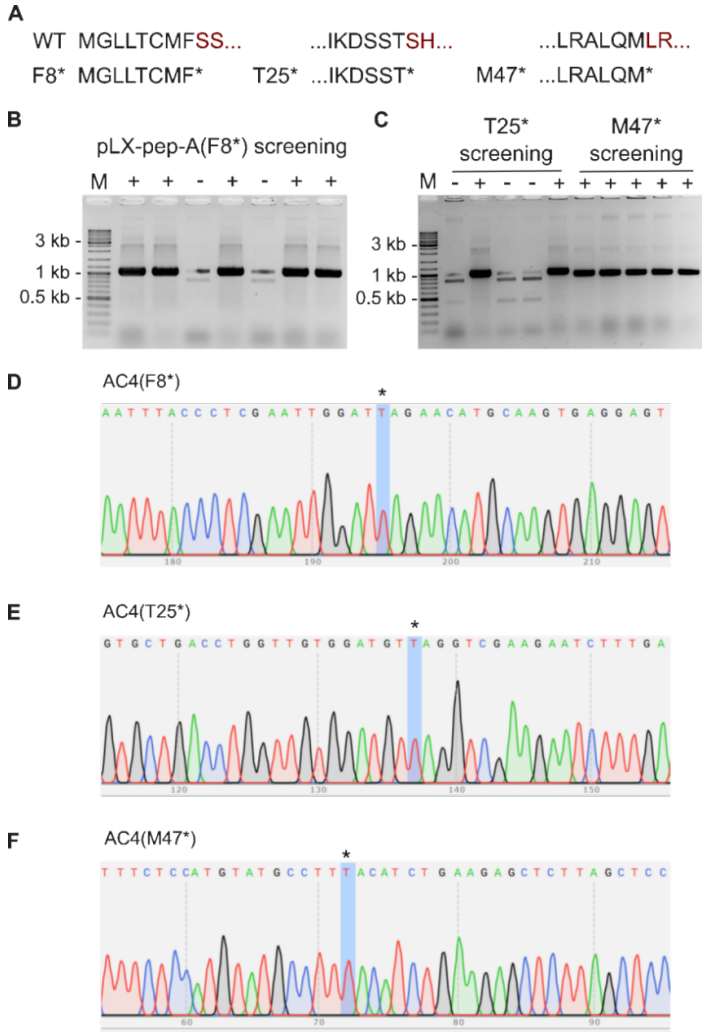

Figure 2. (A) *AC4*-null mutants where a stop codon is inserted at *AC4(F8*)*, *AC4(T25*)* or *AC4(M47*)*. (B-C) PCR with primers capable of binding to the mutant DNA was used to screen for the presence of mutant DNA. The resulting products were 1053 bp, 1002 bp and 936 bp for *AC4(F8*)*, *AC4(T25*)* and *AC4(M47*)* respectively. '+': the presence of the mutant; '-': the absence of the mutant; M: DNA markers. (D-F) DNA sequencing chromatograms showing the nucleotide sequence of *AC4*-null mutants (D) *AC4(F8*)*, (E) *AC4(T25*)* and (F) *AC4(M47*)*. '*' indicates the point mutation

3.2 Agroinoculation of *Nicotiana benthamiana*

The infectious clones were transformed to *Agrobacterium tumefaciens* EHA105 and selected on LB plates containing rifampicin (25 μg/mL) and kanamycin (50 μg/mL). In the absence of the DNA-A infectious clone pLX-Pep-A, *N. benthamiana* inoculated with pLX-Pep-B showed no disease symptoms (Figure 3, mock controls). In contrast, when both pLX-Pep-B and wild-type pLX-Pep-A were applied, disease symptoms appeared five to six days post inoculation and severe leaf distortion was observed at 12 days post inoculation (Figure 3A-B). At 28 days post inoculation, severe leaf distortion was accompanied with white patches on leaves and stunted growth (Figure 3C). These observations were consistent with our previous report using pLX-Pep-A and pLX-Pep-B [19,24]. The DNA-B infectious clone alone could not cause disease symptoms, since efficient replication of viral DNA requires the Rep protein, which is encoded by *AC1* gene on DNA-A. This is consistent with findings in other bipartite begomovirus such as tomato golden mosaic virus, African cassava mosaic virus, abutilon mosaic virus, potato yellow mosaic geminivirus [10,11,25,26].

In *N. benthamiana* infected with pLX-Pep-B and one of the three *AC4*-null mutants, the disease onset also started at five to six days post inoculation and severe leaf distortion was observed at 12 days post inoculation, similar to those infected with the wild-type pLX-Pep-A and pLX-Pep-B (Figure 3A-B). At 28 days post inoculation, we observed symptoms of severe leaf distortion, mosaic and white patches on leaves, and stunted growth (Figure 3C). These symptoms resembled those observed on *N. benthamiana* infected with pLX-Pep-A (wild-type) and pLX-Pep-B. The rates of plants expressing disease symptoms were equal in those treated with wild-type and three *AC4*-null mutant infectious clones (100%, Table 3). Additionally, PCR confirmed the presence of DNA-A (wild-type or *AC4*-null mutants) and DNA-B in symptomatic leaves (Figure 4). Taken together, these results suggested *AC4* plays an insignificant role in the infectivity of PepYLCThV in *N. benthamiana*.

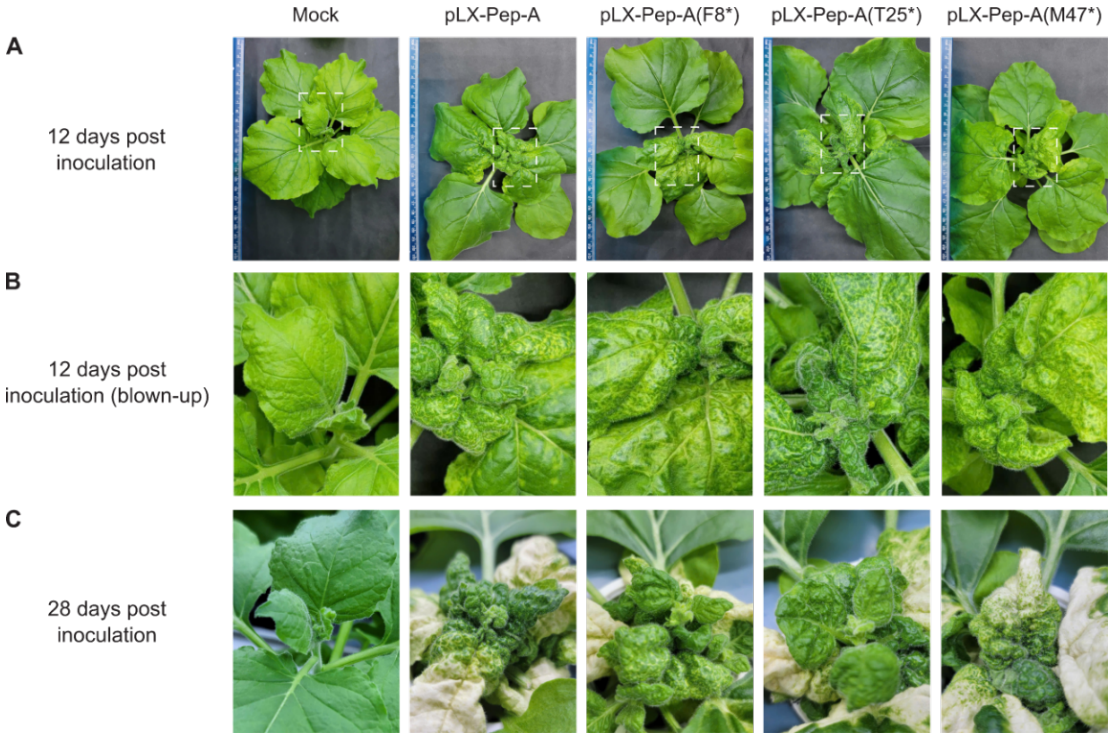

Figure 3. *N. benthamiana* infected with DNA-A and DNA-B infectious clone at (A-B) 12 days or 28 days (C) post inoculation. While pLX-Pep-B was always inoculated, the DNA-A infectious clone was either absent (mock treatment), wild-type (pLX-Pep-A) or *AC4*-null mutant (pLX-Pep-A(F8*), pLX-Pep-A(T25*) and pLX-Pep-A(M47*)). Blue rulers (left edges) in (A) represent 23 cm. White boxes (overlaid) in (A) represent the enlarged areas shown in (B)

pISSN 2588-1191
eISSN 2615-9708

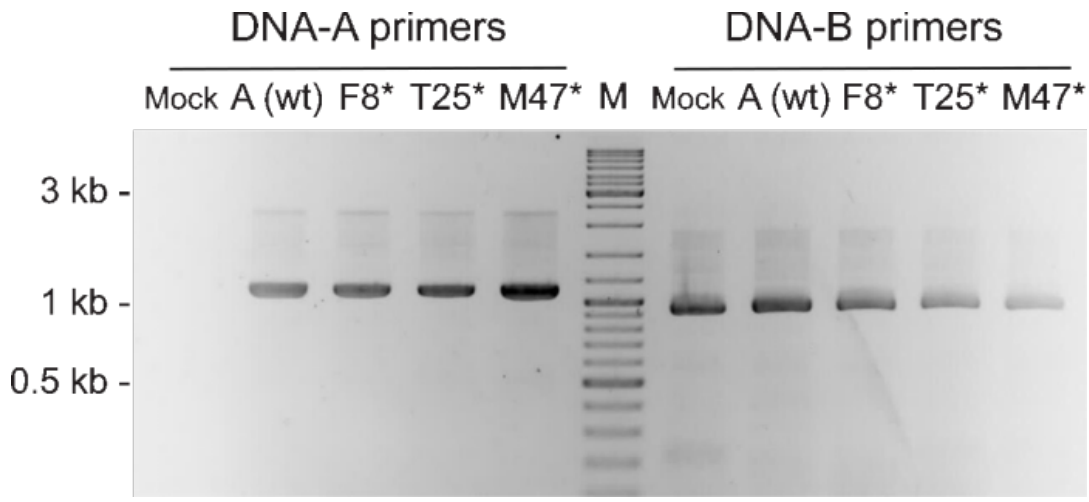

Figure 4. Gel electrophoresis of PCR products using DNA-A and DNA-B primers. Leaf samples were taken at 28 days post inoculation. M: DNA markers.

Table 3. Infectivity of *AC4*-null PepYLCThV infectious clones in *N. benthamiana*. All plants were inoculated with the infectious clone of DNA-B (pLX-Pep-B). In mock treatment, the infectious clones of DNA-A were omitted.

Treatment	Number of plants infected/inoculated			Infectivity (%)	Days when symptoms first observed
	Repeat 1	Repeat 2	Repeat 3		
Mock treatment	0/2	0/3	0/3	0	-
pLX-Pep-A (wild-type)	2/2	3/3	3/3	100	5-6 days
pLX-Pep-A(F8*)	2/2	3/3	3/3	100	5-6 days
pLX-Pep-A(T25*)	2/2	3/3	3/3	100	5-6 days
pLX-Pep-A(M47*)	2/2	3/3	3/3	100	5-6 days

3.3 Discussion

In this work, we created three *AC4*-null mutants of the DNA-A using the QuikChange site-directed mutagenesis method. These mutants were selected to introduce a premature termination codon in *AC4* without changing the amino acid sequence of the overlapping *AC1* gene. All *AC4*-null mutants were able to infect *N. benthamiana* with the infectivity rate similar to wild-type *AC4* (100%, Table 3). These results suggested *AC4* was not essential for PepYLCThV infection in *N. benthamiana*. These outcomes were consistent with previous observations in tomato golden mosaic virus and East African cassava mosaic Cameroon virus (EACMCV) [10,13]. In the previous work by Chen and co-authors (2019), the double mutants of EACMCV *AC4* failed to cause disease symptoms in *N. benthamiana,* leading to the conclusion that *AC4* played a role in EACMCV infection. However, the second mutation in these mutants also resulted in amino acid sequence change in *AC1*, and it remained possible that these unintended changes in *AC1* abolished the inability of mutant clones to cause infection. Further work is required to confirm this hypothesis.

One of the potential explanations for the minor role of PepYLCThV *AC4* in *N. benthamiana* was the functional redundancy with *AC2* protein, both were able to suppress the host plant's RNA silencing defense mechanisms [27,28]. AC2 suppresses RNA silencing mainly through transcriptional regulation of host genes involved in silencing pathways whereas AC4 often acts more directly, interfering with small RNA pathways and interacting with key silencing components [29].

A limitation of this work is the use of *N. benthamiana* as the experimental host. While *N. benthamiana* is frequently used as a model organism in plant virology due to the ease of infecting this plant species [30], their compromised defence system may obscure potential roles of AC4 in helping PepYLCThV to evade the plant defence system. Hence, further work needs to be performed with *AC4*-null mutant infectious clones and PepYLCThV natural host *C. annuum* to more comprehensively determine the role of AC4. Furthermore, in addition to morphological observations, viral load quantification will be useful to compare the effects of various *AC4*-null infectious clones.

4 Conclusion

In this work, we have created three *AC4*-null mutants of the DNA-A PepYLCThV infectious clones. We found that these mutants retained the ability to cause severe leaf curl in *N. benthamiana*, with infectivity rate and disease severity similar to wild-type DNA-A. Hence, the *AC4* gene appeared to play a minimal role in the infectivity of PepYLCThV in *N. benthamiana*. Further studies are required to determine whether AC4 plays a role in the infectivity of PepYLCThV in the natural host *C. annuum*.

Acknowledgement

The authors are thankful to the Ministry of Education and Training of Vietnam (grant no. B2024-DHH-10) for the financial support to conduct the current research. The authors also acknowledge the partial support of Hue University under the Core Research Programme (grant no. NCTB.DHH.2024.03), which covers article processing charges. We thank Nguyen Le Quy Bao for help with DNA extraction and PCR, Dr. Nguyen Huu Kien and Dr. Do Tien Phat for A. tumefaciens EHA105 and N. benthamiana seeds respectively.

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