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RESEARCH ARTICLE

Symptom Severity of *Nicotiana benthamiana* Plants Inoculated with *Agrobacterium* Containing Infectious DNA-A Clones of *Honeysuckle Yellow Vein Virus* (HYVV)

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ABSTRACT

To investigate the pathogenicity and virulence of the *Honeysuckle yellow vein virus* (HYVV) lacking betasatellites, PCR amplified unit-lengths of DNA-A genome of HYVV-[DJ] were cloned into binary vector pRI101-AN, and generated HYVV-[DJ]-1mer, -1.3mer and -2mer genomes. Each construct was transformed into *Agrobacterium* cells and agro-inoculated into young leaves of *Nicotiana benthamiana*. Except for the HYVV-[DJ]-1mer, HYVV-[DJ]-1.3mer and -2mer clones caused pronounced disease symptoms in *N. benthamiana*. HYVV-[DJ]-2mer agro-inoculated plants showed more severe plant stunting with downward leaf curling and crinkling than those of HYVV-[DJ]-1.3mer agro-inoculated plants. To discriminate the clone's virulence quantitatively, SYBR Green-based real-time PCR was performed for the quantification of the target virulence gene DNA in agro-inoculated plants that were collected at weekly intervals for 4 weeks. Regression analysis was obtained from the standard curves by plotting Ct values over the logarithm of the amount of V1 protein gene DNA present in a dilution series of plasmid containing the full-length HYVV-[DJ] genome. Equation of the HYVV *V1* DNA standard curve was used to quantify *V1* gene DNA concentration in agro-inoculated plants with each clone. The accumulation of *V1* gene DNA in HYVV-[DJ]-1.3mer agro-inoculated plants reached the peak level at 4 weeks post inoculation, while the accumulation of *V1* gene DNA in HYVV-[DJ]-2mer agro-inoculated plants reached the peak level at 3 weeks post inoculation. The amount of *V1* DNA in HYVV-[DJ]-1.3mer agro-inoculated plants was significantly more than that in HYVV-[DJ]-2mer agro-inoculated plants. Considering the results, there was a difference between the accumulation of virus DNA and the symptom severity of the analyzed plants agro-inoculated with each clone. It suggested that the infectious clones' virulence is not necessarily correlated with the symptom severity.

Keyword: *Honeysuckle yellow vein virus* (HYVV), Symptom severity, Virulence of HYVV DNA-A clones, *V1* gene, SYBR Green-based real-time PCR

1 Introduction

In Korea, tomato (*Solanum lycopersicum*)-infecting begomoviruses (family *Geminiviridae*) have become widely spread nationwide over the years [1-3]. Among these viruses, *Honeysuckle yellow vein virus* (HYVV) infects tomato plants, causing symptoms such as small leaves showing yellowish edge and plants showing short internodes and stunted growth [2]. HYVV also induces yellow vein symptoms accompanied by oval-shaped enations on the bottom side of leaves of the natural host, *Lonicera japonica* (honeysuckle) [4]. Genome sequences of HYVV have been reported from the United Kingdom, Japan and Korea [2, 5, 6]. In Korea, two HYVV isolates, HYVV-[Jeju] (accession number FJ434943) and HYVV-[DJ] (accession number HQ189431),

were identified from tomato plants [2], while a HYVV isolate, HYVV-[Kr] (accession number GQ477135), was identified from symptomatic leaves of *L. japonica* [5]. The analysis of HYVV-[Jeju] and HYVV-[DJ] revealed that they are distantly related to known HYVV isolates. Interestingly, HYVV-[Jeju] and HYVV-[DJ] are not associated with betsatellites [2], while HYVV-[Kr] is associated with betasatellites (accession number GQ495268) [5]. In addition, HYVV-[Jeju] and -[DJ] are close to HYVV-[Masuda] (89.4-92.8% nt identity), while HYVV-[Kr] is close to HYVV-[UK2] based on nucleotide (nt) sequence homology and phylogenetic tree analysis.

Conventional virus detection in virus-infected plants was attempted mostly to detect viral genes or genomes by polymerase chain reaction (PCR)-based methods or antibody-based serological assays. PCR is the most rapid and sensitive technique for diagnosis using primers designed from specific begomovirus sequences [3, 7-9]. These PCR primers allow the virus to be detected qualitatively but cannot quantify the virus titer in infected plants. The use of real-time PCR to quantify the infection of a plant by a virus has increased over the years. Recently, a real-time TaqMan PCR method was used for the diagnosis, discrimination and quantification of *Tomato yellow leaf curl virus* (TYLCV) and/or the *Tomato yellow leaf curl Sardinia virus* (TYLCSV) in tomato plants or whiteflies [10-12]. The real-time TaqMan PCR method is based on the hydrolysis probes that labeled with a fluorophore at one end and a quencher at the other end and this method requires a high complementation in the probe-binding site [13, 14]. In contrast, SYBR Green dye binds non-specifically to the double-stranded DNA by intercalation [15, 16]. Although these two methods are fast, sensitive and have potential for multiplexing, the SYBR Green-based real-time PCR method is simpler and cheaper than the real-time TaqMan PCR method.

The aim of this work was to understand the effect of three clones (HYVV-[DJ]-1mer, -1.3mer and -2mer) on symptom severity and virus DNA accumulation in *Nicotiana benthamiana* plants. Therefore, we inoculated *N. benthamiana* plants with *Agrobacterium* containing each clone and observed symptom development for 4 weeks. To estimate HYVV *V1* gene DNA accumulation in *N. benthamiana* plants, we performed a SYBR Green-based real-time PCR assay to quantify HYVV virulence by the specified *V1* gene fragment.

2 Materials and Methods

2.1 Vector Construction for Infectious Clones

A recombinant vector (pGEM-T Easy) containing HYVV-[DJ] full-length genome [2] was used as a primary template for the construction of three infectious DNA-A clones in this study. Firstly, a unit of full-length genome (1mer) was amplified using a set of genome-specific primers (Table 1); HYVV-1merF containing *Sal*I site (forward primer) and HYVV-1merR containing *Eco*RI site (reverse primer) as follows.

Table 1: *List of Honeysuckle yellow vein virus (HYVV) genome- and gene-specific primers used in PCR and real-time PCR*

Reaction	Primers	Nucleotide sequences (5'-3')
PCR	HYVV-1merF[a]	ACGCGTCGACATGTGGGATCCTTTA
	HYVV-1merR[b]	GAATTCCTCACACCGTTACAATTAGGGCT
	HYVV-0.3merF[c]	GGAATTCATGTGGGATCCTTTA
	HYVV-0.3merR[d]	GGAATTCTTAATTCTGTACTGAATCATAGAAATACACTCT
	HYVV-V1F	ATGTCGAAGCGWCCA
	HYVV-V1R	TTAATTTKRTGAATCATAGAA
Real-time PCR	HYVV-RT-V1F	ATGTCGAAGCGTCCAGCAGATAT
	HYVV-RT-V1R	AGCGCACGGTTGGCATACG
	N. benthamiana 5.8s rRNA-F	GTGATCTGTGGAAGGATCATTGTCG
	N. benthamiana 5.8s rRNA-R	CGTTAATCATCCGACACGAACGC

[a] Forward primer contains *Sal*I site (underline). [b] Reverse Primer contains *Eco*RI site (underline).
[c] Forward primer contains *Eco*RI site (underline). [d] Reverse Primer contains *Eco*RI site (underline).

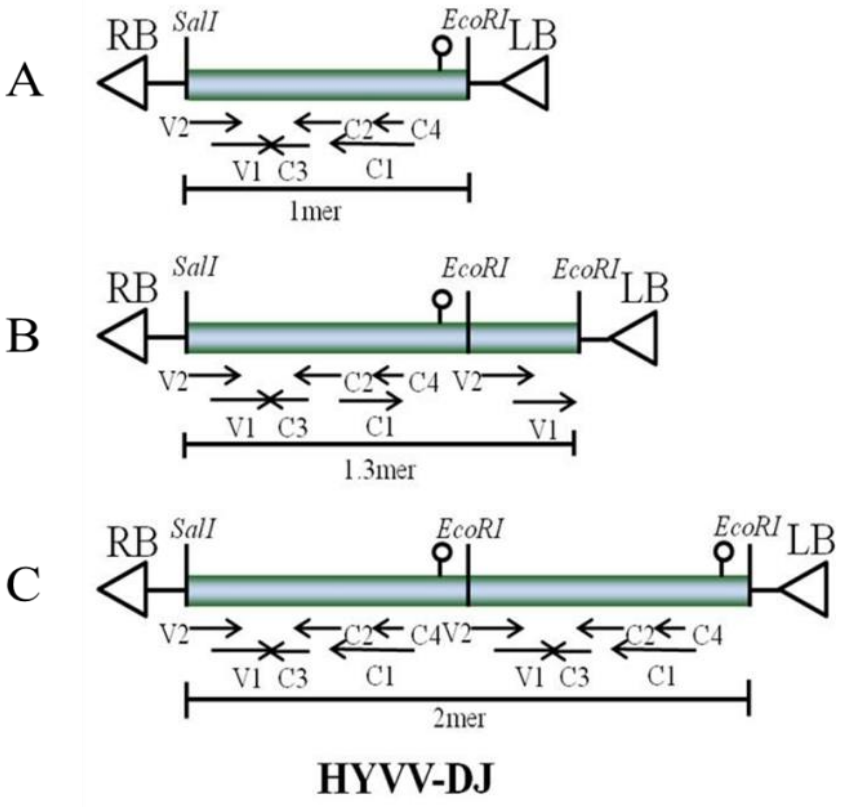

Figure 1: *Diagram of the plasmid constructs of three infectious clones of HYVV-[DJ] DNA-A. The open circles indicate the stem-loop-forming region. The arrows represent predicted open reading frames in both orientations (V for viral sense and C for complementary sense). The restriction sites were used for cloning. Constructs from top to bottom are 1mer (A), 1.3mer (B) and 2mer (C) of HYVV genome.*

PCR was conducted in reaction mixture (50 µl) consisting of 10x PCR buffer (100 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$ and 500 mM KCl, Takara, Japan), 10 ng of template DNA, 4 µl of 2.5 mM deoxynucleotide triphosphates (dNTP), 1 µl (50 pM) of primer each, 1.5 mM of $MgCl_2$ and 2.5 U of Ex-*Taq* polymerase (Takara, Japan). The PCR was conducted in a thermal cycler (Bio-Rad, USA) with 1 cycle of denaturation at 95°C for 2 min and followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 2 min and extension at 72°C for 3 min and additional 1 cycle of final extension at 72°C for 7 min. The target size of band in 1% agarose gel was purified using the PCR quick-spin™ (Intron, Korea) according to the manufacturer's protocol, double-digested with *Sal*I and *Eco*RI and cloned into a pRI101-AN vector (Takara, Japan). The resultant vector was named pRI-HYVV-1mer (Fig. 1A). Secondly, a partial genome of 0.3mer (829 bases) fragment enclosing HYVV-V1 and -V2 protein coding regions was amplified using a set of primers; forward primer HYVV-0.3merF containing *Eco*RI site and reverse primer HYVV-0.3merR containing *Eco*RI site under the same PCR conditions above. The PCR-amplified 0.3mer product was digested with *Eco*RI and then ligated with *Eco*RI-digested pRI-HYVV-1mer. The resultant vector was named pRI-HYVV-1.3mer (Fig. 1B). To make two copies of the HYVV genome in a vector, the PCR-amplified 1mer product was digested with *Eco*RI and ligated with *Eco*RI-digested pRI-HYVV-1mer to generate pRI-HYVV-2mer (Fig. 1C). To generate infectious HYVV clones, *A. tumefaciens* C58C1 was transformed with pRI-HYVV-1mer, pRI-HYVV-1.3mer and pRI-HYVV-2mer, respectively.

2.2 Agro-inoculation into *N. benthamiana* by Syringe Infiltration

To confirm that the HYVV clones are infectious without betasatellite association, *Agrobacterium* containing each HYVV clone was inoculated into

lower leaves of *N. benthamiana* plants using a needleless syringe. Fifty-four plants (6 plants/clone, 3 repeats) were monitored for symptom development and symptom severity at weekly intervals for 4 weeks post inoculation.

2.3 Comparison of Sensitivity between Conventional PCR and Real-time PCR

To compare the detection limit of two assays, the plasmid containing the full-length HYVV-[DJ] genome was prepared as a standard DNA after serial 10-fold dilutions (1-10^{-9} ng/µl) of the plasmid DNA in nuclease free water. The diluted standard plasmid DNA was tested with PCR using HYVV *V1* gene-specific primers (Table 1). PCR was conducted under the same conditions described in section 2.1, except for 10-fold diluted template DNA and 40 cycles of annealing at 56°C for 2 min. The SYBR Green-based real-time PCR amplification was conducted in 96-well plates by a Stratagene Mx3005P cycler. The reaction mixture (20 µl) contained 10-fold diluted template DNA, 10 µl of 2x SYBR Green QPCR Master Mix (Agilent Technologies, USA), 1 µl of primer pairs at 10 pM and 7 µl of distilled water. For the real-time PCR assay, primer sets (Table 1) were designed to cover a 96 nt region in the *V1* gene. The reaction was carried out under the following conditions: 1 cycle of initial denaturation at 95°C for 15 min, 40 cycles of denaturation at 95°C for 10 s, and annealing and extension at 60°C for 40 s. The experiments were analyzed with auto-baseline and manual thresholds chosen from the exponential phase of the PCR amplification. After the data analysis, the Ct number and DeltaRn (dRn) were used for statistical analyses. Data were analyzed using Mxpro software and the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method [17].

2.4 PCR Detection of *V1* Gene in Agro-inoculated Leaves

Leaves were harvested from three different plants agro-inoculated with each clone. Total DNA was extracted from leaf tissues using the DNeasy Plant Mini kit (Qiagen, German) according to the manufacturer's directions. The presence of viral DNA in the leaf tissues was confirmed by PCR using HYVV *V1* gene-specific primers such as HYVV-V1F and HYVV-V1R (Table 1). PCR was conducted under the previously described conditions in section 2.3, except for 100 ng of template DNA.

2.5 Quantification of HYVV Virulence by SYBR Green Real-time PCR

The SYBR Green-based real-time PCR amplification was performed under the described conditions above, except using 1 µl of total DNA (1 ng/µl) extracted from agro-inoculated leaf tissues as a template DNA. To determine the reproducibility of the assay, total DNA was extracted from the most upper leaf of 3 different plants agro-inoculated with each clone. Each sample was quantified in triplicate and processed three different times with real-time PCR under the same conditions. For the generation of a standard curve, the Ct values were plotted proportionally to the logarithm of the input plasmid DNA concentration that was diluted from 10^{-1} to 10^{-9} ng/µl plasmid DNA. Negative controls, such as no template control and DNA extracted from mock-infiltrated *N. benthamiana* of the same developmental stage, were simultaneously included in each run. Quantification of a relative amount of respective HYVV *V1* DNA in the agro-inoculated plants with each clone was calculated by comparing the Ct value of each sample to the Ct values of standard curve. As Ct values may vary slightly between experiments, we included one dilution series of standard DNA in each plate, obtaining one regression line per plate. Thus, the amount of starting template in any real-time PCR reaction, expressed as ng of viral gene DNA per 1 µg of total DNA in the original plant extract, could be accurately determined. These values were used as estimates of the virulence of each clone.

2.6 Statistical Analysis of Data

Data were analyzed for statistical significance by SAS (Statistical Analysis System, version 9.1, Cary, NC, USA), and means were compared using Duncan's multiple range tests. Graphing was conducted with SigmaPlot 10.0 (Systat Software, Inc., San Jose, CA, USA).

3 Results and Discussion

3.1 Symptom Severity of HYVV Clones using Agro-inoculation into *N. benthamiana*

Initial symptoms appeared in 7 days post inoculation with HYVV-[DJ]-2mer and in 7-12 days post inoculation with HYVV-[DJ]-1.3mer, respectively. HYVV-[DJ]-2mer agro-inoculated plants showed more severe symptoms and stronger intensity of viral band than those of HYVV-[DJ]-1.3mer agro-inoculated plants (Fig. 2A, B and C). All assayed plants were susceptible to two clones and no immune or hypersensitive resistance responses were observed. Two clones caused plant stunting with downward leaf curling and crinkling, but they showed different degree of symptom severity. It suggested that HYVV DNA-A genome can cause symptoms without association of betasatellites. In an earlier study [5], *N. benthamiana* plants inoculated with HYVV-[Kr]-1mer did not show any symptoms. Furthermore, no viral band was observed by Southern blot hybridization. In this study, HYVV-[DJ]-1mer clone agro-inoculated plants remained asymptomatic for 4 weeks post inoculation like mock inoculated plants (Fig. 2A and C). Besides, no viral DNA band was observed in PCR (Fig. 2B, lane 2).

It is known that a viral genome DNA can be synthesized, spread systemically throughout the plant and can induce disease symptoms by the agro-inoculation method [18]. An excised unit-length DNA or a cloned DNA containing a single copy of a certain geminivirus genome was infectious using biolistic inoculation [19], while most infectious clones required (partially or completely) tandem-repeat constructs of geminivirus genome to enhance their infectivity by agro-inoculation [20].

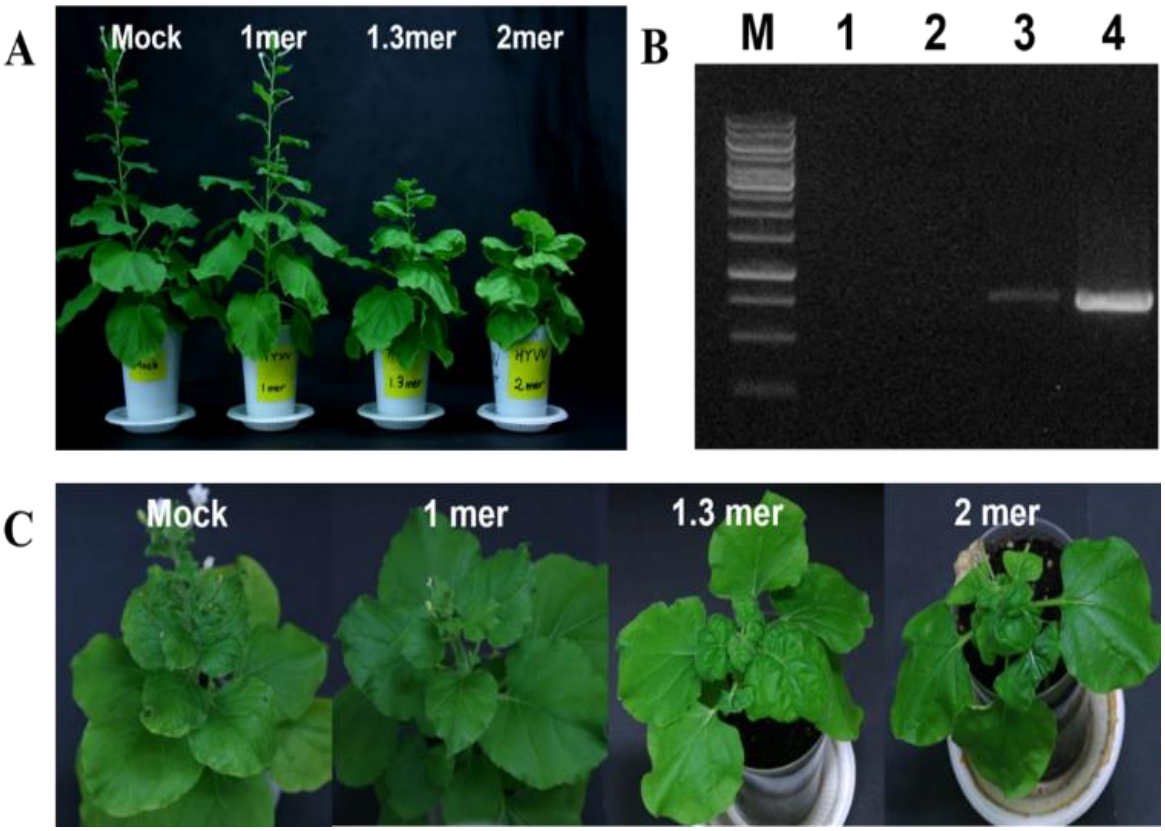

Figure 2: *(A) Stunting symptom of N. benthamiana plants at 4 weeks post agro-inoculation with mock, pRI-HYVV-1mer, -1.3mer and -2mer, respectively. (B) The presence of V1 DNA in the leaf tissues agro-inoculated with clones was confirmed using HYVV V1 gene-specific primers. (C) Lanes 1-4: mock inoculated, pRI-HYVV-1mer, -1.3mer and -2mer. Leaf symptoms of N. benthamiana agro-inoculated with each clone showing crinkling and downward curling leaves.*

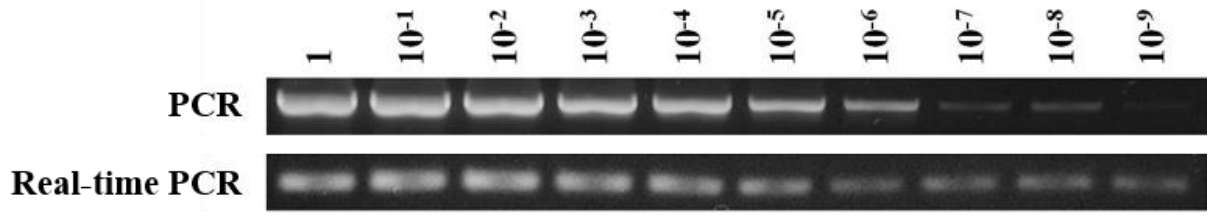

Figure 3: *Agarose gel (1.5%) shows detection limit of V1 gene amplified from pRI-HYVV-1mer by PCR and SYBR green real-time qPCR. Lanes 1-10: 10-fold serial dilutions of the recombinant plasmid (1-10^{-9} ng/µl DNA).*

3.2 Selection of Target Genes and Sensitivity Comparison to Detect Target Genes between PCR and Real-time PCR

To measure the virulence of each clone quantitatively, we considered virulence factors such as viral proteins coded by virulence genes. As a marker we targeted a specific gene that displays diverse functions in virus-plant interactions. The open reading frame (ORF) V1 is a coat protein that is involved in cell-to-cell movement [21], particle assembly [22], insect transmission [23], nucleo-cytoplasmic shuttling within the host cell and DNA-binding [24]. To determine the detection end-point of HYVV *V1* gene between two different assays, the sensitivity of real-time PCR was compared with that of conventional PCR using serial dilutions of plasmid DNA. In a PCR experiment using HYVV *V1* gene-specific primers, the amplified products of *V1* gene DNA were barely detectable in a sample containing 10^{-8} ng of plasmid DNA. In a real-time PCR, the amplified products of *V1* gene DNA were clearly detectable in a sample containing only 10^{-9} ng of plasmid DNA (Fig. 3). Thus, real-time PCR appears to be more sensitive than PCR under the given experimental conditions.

3.3 Standard Curve for Real-time PCR

Since HYVV V1 protein is related to virulence, the quantification of *V1* gene DNA in agro-inoculated plants was performed using real-time PCR analysis. The standard curve showed a high correlation coefficient and a linear relationship between Ct values and the logarithm of the starting template amounts over the range of examined DNA concentrations (Fig. 4A).

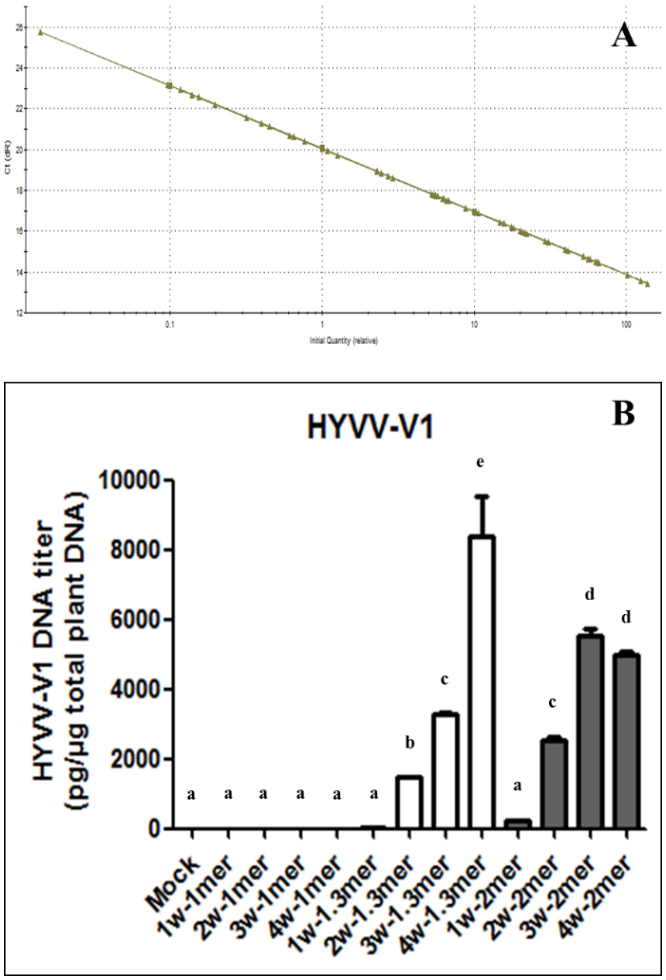

Figure 4: *Standard regression line obtained by plotting Ct values versus the logarithm of the amount of HYVV V1 DNA (A). DNA standard samples were prepared by 10-fold diluting a full-length genomic clone of HYVV-[DJ] from 10^{-1} to 10^{-9} ng/µl DNA. Average amount of V1 DNA quantified by SYBR Green real-time PCR in leaf tissues of 3 different plants of N. benthamiana at 1, 2, 3 and 4 weeks post agro-inoculation with pRI-HYVV-1mer, -1.3mer and -2mer, respectively. Mean separation by Duncan's multiple range test at P = 0.05. The same letter above bars represented no significant difference between treatments.*

As expected, the Ct value decreased as the starting DNA concentration increased. The ten-fold dilution series made it theoretically possible to quantify *V1* gene DNA concentrations. Equation obtained on the standard curves by plotting the Ct values over the logarithm of the amount of *V1* gene DNA present in plasmid dilution was $y = -3.081\log(x) + 20.05$; $r^2 = 1.000$ and efficiency 111%. Regression analysis of the standard curve indicated that the assay is highly efficient and can be used to quantify *V1* gene DNA concentration in agro-inoculated plants with each clone.

3.4 Quantification of HYVV Infectivity in Agro-inoculated *N. benthamiana* by Real-time PCR

The standard curve allows for the quantification of the HYVV *V1* gene DNA concentration in 1 µg of total plant DNA extracted from the leaf tissues of agro-inoculated plants. The quantity of *V1* gene DNA in a sample was determined by comparing its Ct value with that of the standard curve for absolute quantification of the viral gene DNA. No signal was detected from total genomic DNA of mock-inoculated control plants and negative control. However, the *V1* gene DNA accumulation could be detected reproducibly in some agro-inoculated plant using real-time PCR with primer sets of *V1* gene. Significant differences in the accumulated *V1* gene DNA were found between the analyzed clones, indicating differences in *V1* gene DNA accumulation that correlated to their different levels of virulence.

Like mock-inoculated plants, the accumulation of *V1* gene DNA in HYVV-[DJ]-1mer agro-inoculated plants was negative during the experimental period (Fig. 4B). In HYVV-[DJ]-1.3mer agro-inoculated plants, the accumulation of *V1* gene DNA was barely detectable at 1 week post inoculation and reached the peak level at 4 weeks post inoculation. In HYVV-[DJ]-2mer agro-inoculated plants, the accumulation of *V1* gene DNA was weakly detected at 1 week post inoculation and reached the peak level at 3 weeks post inoculation. At 4 weeks post inoculation, infected plants with the HYVV-[DJ]-2mer clone showed more severe symptoms and significantly less *V1* gene DNA accumulation than infected plants with the -1.3mer clone. Considering the obtained results, the accumulation level of the *V1* gene DNA was not positively correlated with the symptom severity displayed by the source plant.

Some studies of plant-virus interactions exhibited a positive correlation between symptom severity and a viral protein accumulation [25, 26], while others have failed to detect a link between symptom severity and virus accumulation [27, 28]. Additionally, resistance breaking isolate did not result in high virus accumulation in the infected plants [29], indicating that the correlation between viral accumulation and virulence is not evident [30]. It suggests that more severe symptoms in plants provide less favourable conditions to replicate virus particles.

4 Conclusions

It is known that monopartite begomoviruses are usually associated with betasatellites that are essential for induction of typical disease symptom. The present study concludes that HYVV clones are highly infectious to *N. benthamiana* plants, even without association of betasatellites. Additionally, the study revealed that the agro-inoculated plants with a HYVV-[DJ]-2mer clone showed more severe symptoms and less accumulation of *V1* gene DNA than those agro-inoculated with a HYVV-[DJ]-1.3mer clone at 4 weeks. These results provide additional evidence that the symptom severity may not necessarily be strongly tied with virus accumulation in a host plant.

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