Vietnam Journal of Biotechnology **21**(3): 513-529, 2023

EXPRESSION OF GLOBAL CONSENSUS NON-STRUCTURAL PROTEIN 1 (NS1) OF DENGUE VIRUS SEROTYPES 1 AND 2 IN *NICOTIANA BENTHAMIANA*

Cu Le Nguyen[1,†], Nguyen Ngoc Luong[2,†,✉], Tuong Quoc Trieu[3], Nguyen Hoang Loc[2]

[1]*Buon Ma Thuot Medical University, 298 Ha Huy Tap Road, Tan An Ward, Buon Ma Thuot City, DakLak Province, Vietnam*
[2]*Department of Biology, College of Sciences, Hue University, 77 Nguyen Hue Road, Phu Nhuan Ward, Hue City, Thua Thien Hue Province, Vietnam*
[3]*Tay Nguyen Institute of Hygiene and Epidemiology, 34 Pham Hung Road, Tan An Ward, Buon Ma Thuot City, DakLak Province, Vietnam*

[†]These authors have contributed equally to this work

[✉]To whom correspondence should be addressed. E-mail: luongnguyenbio@hueuni.edu.vn

Received: 13.6.2023
Accepted: 20.8.2023

SUMMARY

Dengue viruses, which consist of four distinct serotypes, belong to the Flavivirus family, one of the most important arthropod-borne pathogen groups for humans. Vietnam is among the tropical countries that have endemic dengue outbreaks, and in 2022, the number of infections as well as fatal cases reached a record high, causing a great economic burden to the public health system. Although all four dengue serotypes have been circulating in Vietnam, serotypes 1 and 2 have been dominating in the last few decades. Since most dengue clinical complications result from secondary infection with a different serotype from the first infection, prior knowledge of the proportion of a population having seropositive to a certain serotype is very crucial for public health policy makers. The absence of comprehensive and regular serological epidemiological surveys in Vietnam has impeded progress in surveillance and the implementation of effective preventive measures. To facilitate research into dengue treatment and diagnosis, we created two synthetic, codon-optimized genes encoding for global consensus non-structural protein 1 (NS1) from dengue serotypes 1 and 2. We expressed these genes in *Nicotiana benthamiana* using the agroinfiltration technique. Western blot using Dengue patients' sera indicated that the target proteins were expressed, likely in both mono and multimeric forms. Our results indicated that *N. benthamiana* is a good alternative to other methods of producing recombinant NS1.

Keywords: *Agrobacterium tumefaciens*, agroinfiltration, consensus, dengue virus, non-structural protein 1, serotype 1, serotype 2

INTRODUCTION

The Flaviviridae family includes viruses with a genome of positive single strand RNA of approximately 10 kb. The most significant genus, *Flavivirus*, consists of some of the most important arthropod-borne viruses that cause diseases in humans, such as yellow fever, West Nile, Murray Valley, Zika, dengue fever, and Japanese encephalitis (Pierson, Diamond, 2013). Among the flaviviruses, dengue viruses are the most significant due to their global distribution and the number of countries with endemic dengue fever (Guzman *et al.,* 2010). Annually, dengue viruses are responsible for over 100 million infection cases, of which more than 500,000 were admitted to hospitals and between 10,000 to 25,000 ended with deaths (Cattarino *et al*., 2017). In 2022, Dengue cases surged around the world, reached a record high since the global peak in 2019 (Taylor, 2023). Among countries affected, Vietnam was ranked second in terms of the number of dengue cases per 100,000 population (WHO, 2022).

Based on serological assays, dengue viruses are classified into four serotypes: dengue virus serotype 1, dengue virus serotype 2, dengue virus serotype 3, and dengue virus serotype 4 (abbreviated as DENV1, DENV2, DENV3, and DENV4) (Messina *et al*., 2014). However, owing to their rapid evolution and extensive variability, dengue viruses have been further categorized into genotypes, strains, and quasispecies (Pierson, Diamond, 2013). Moreover, an in-depth analysis of antigen variability within serotypes revealed that the canonical serotypes are not antigenically homogenous, as viruses within the same serotype may be as different as viruses from different serotypes (Katzelnick *et al*., 2015). As for Dengue antigenic evolution, over the past 20 years, it has been shown that although sequences from different serotypes tend to become more and more different over a long period of time, for brief periods when sequences of different serotypes are more similar to one another, they coincide with an increase in epidemic severity (Katzelnick *et al*., 2021).

Dengue infection usually results in a mild fever but may lead to fatal complications, which include dengue hemorrhage fever and dengue shock syndrome (Guzman *et al*., 2010). The severe forms of dengue fever are assumed to be the result of secondary infection with a distinct serotype, in which preexisting antibodies not only fail to neutralize the current virus but actually enhance its ability to infect important immune cells. This phenomenon is termed antibody enhancement and has been implicated in mechanisms of vascular leakage and cytokine storms (Pang *et al*., 2006; Katzelnick *et al*., 2017, Sarker *et al*., 2023). The dengue virus heterogenicity and antibody enhancement are the main roadblocks toward developing effective vaccines against Dengue. While vaccines from reputable sources such as Sanofi, NIAID, and Takeda have recently been approved, unfortunate incidents during their implementation in children of certain countries have raised significant concerns, thus hindering the widespread adoption of these vaccines (Foucambert *et al*., 2022).

To cope with the problem of dengue virus variability, vaccine and diagnostic tool development have been utilizing a very common approach: searching for more conserved antigens among the viruses or trying to create consensus antigens that represent the most common antigenic features of each serotype or all of the

serotypes. The second approach has been widely used to create consensus antigens for important antigens such as non-structural protein 1 (NS1) (Park *et al*., 2020) and envelope protein (E) (Leng *et al*., 2009; Danecek *et al*., 2010; Hussain *et al*., 2015; Huang *et al*., 2022; Sankaradoss *et al*. 2022). Though different in approaches, the main theme for consensus antigen creation is to search for commonalities within variabilities. For example, Leng *et al*. (2009) simply created consensus sequences for each serotype and then used the consensus sequences of four serotypes to create a consensus sequence for all dengue viruses. On the other hand, Park *et al*. (2020) created a database of epitopes from NS1, built phylogenetic trees, and then created the consensus proteins.

NS1 plays multiple roles in the pathogenesis of dengue and is the main target for vaccine and diagnostic tool development for flavivirus in general and dengue in particular (Glasner *et al*., 2018). To address the problem of dengue virus heterogeneity within each serotype, we created the consensus sequences of NS1 of dengue virus serotypes 1 and 2, which represent the most commonly encountered variants found in the NCBI Genpept database. It was reported that in Vietnam, these two serotypes are the most prevalent and widespread (Nguyen *et al*., 2019). Using the consensus sequences, we predicted the epitopes and matched them with verified epitopes on IEDB (https://www.iedb.org). Subsequently, we codon optimized the genes encoding for these NS1 to be expressed in *Nicotiana benthamiana*, a very popular host for large-scale production of recombinant proteins in native form (Bally *et al*., 2018). To check the expression of the target proteins in *N. benthamiana*, we used protein extraction as the capturing antigen for specific IgG antibodies from Vietnam dengue patients' sera. This report presented our initial findings of producing consensus Dengue NS1 from serotype 1 and serotype 2 in *N. benthamiana*.

MATERIAL AND METHODS

Strains and culture conditions

All of the cloning work was conducted in *E. coli* TOP10, which is maintained in Luria Broth (LB) and LB agar. *E. coli* BL21 De3 Codonplus RIPL expressing NS1 from dengue virus serotypes 1 and 2 were cultured in LB supplemented with chloramphenicol (CAM, 34 µg/mL), streptomycin (Strep, 50 µg/mL) and ampicillin (Amp 50 µg/mL). *Agrobacterium tumefaciens* strain LBA4404 was used for agroinfiltration and normally maintained in LB. *A. tumefaciens* carrying the expression plasmids was maintained in LB supplemented with kanamycin (Kan, 50 µg/mL).

Acquisition of codon-optimized genes for *N. benthamiana* encoding for consensus NS1 from dengue virus serotypes 1 and 2

To obtain dengue virus serotype 1 and 2 consensus NS1 sequences (referred to as DENV1-NS1 and DENV2-NS1), we searched for all of the polyprotein sequences of dengue virus serotypes 1 and 2 from the NCBI Genpept database using BLAST. Retrieved sequences were downloaded in fasta format, and sequences with ambiguous amino acids (such as X) were removed. Subsequently, the remaining sequences were aligned using the MUSCLE algorithm (Edgar, 2004) in MEGA11 (Tamura *et al*., 2021). The alignment results were submitted to

EMBOSS Cons (https://www.ebi.ac.uk/Tools/msa/emboss_cons/) to generate the consensus sequences. The consensus sequences were subsequently used as queries to search for identical homologous proteins in the NCBI non-redundant protein database to ensure the existence of such sequences in nature.

From the consensus DENV1-NS1 and DENV2-NS1 amino acid sequences, we generated several *N. benthaminana* codon optimized genes using the IDT codon-optimization tool (https://sg.idtdna.com/Codon Opt). We selected one sequence for each NS1 based on three criteria: Codon Adaptation Index (CAI), mRNA secondary structure, and 3rd position GC content, which were calculated using Visual Gene Developer (Jung, McDonal, 2011). Gene sequence synthesis was ordered at Phusagenomics (https://phusagenomics.com). The synthetic *N. benthamiana* genes came as cloned genes in pUC19 and are hereinafter referred to as *Denv1-NS1* (accession number: OR349280, encoding for DENV1-NS1) and *Denv2-NS1* (accession number: OR349282, encoding for DENV2-NS1). The genes contain *Xba*I and *SacI*I at the 5' and 3' termini for cloning into pMYV498 (Nguyen *et al*., 2016) to generate two expression vectors: pMYVD1 and pMYVD2. There is a stop codon before *Sac*I site on each gene.

Construction of pMYVD1 and pMYVD2

The pMYV498 carried a Dengue Envelope domain III protein fused with the subunit B of cholera (Nguyen *et al*., 2016), which contains the promoter pd35S (duplicated CaMV 35S promoter), and the original Kozak sequence at the N terminus, and a SEKDEL signal peptide for endoplasmtic reticulum retention (see Fig. 1). The *Denv1-NS1* and *Denv2-NS1* gene sequences were released from pUC19 by *Xba*I/*Sac*I and cloned into pMYV498, which had been linearized using the same pair of restriction enzymes. The recombinant pMYV498 still uses the pd35S promoter to direct the expression of downstream genes, but without an ER retention signal. The resulting recombinant pMYV498 carrying *Denv1-NS1* and *Denv2-NS1* were renamed pMYDV1 and pMYVD2, respectively.

Agroinfiltration procedure

We followed the protocol set up by Nguyen *et al.* (2016) for agroinfiltration. Briefly, *N. benthamiana* seeds were germinated for 2 weeks until seedlings appeared. The seedlings were transferred into flowerpots, with one seedling per pot. The plant was allowed to grow at 25 ºC, under 16-hour light/8-hour dark cycles for another six weeks.

Agrobacterium tumefaciens strain LBA4404 was transformed with pMYDV1 and pMYDV2 together with the helper plasmid pRK2013 using the tri-parental mating method described previously (Horsch *et al*., 1985). pMYV508, carrying the p19 protein of the tomato bushy stunt virus, was also transformed into *A. tumefaciens*. The p19 protein role is to suppress post-transcription RNAi gene silencing, which was shown to significantly improve the expression of the target proteins (Peyret, Lomonossoff, 2015; Nguyen *et al*., 2016; Nguyen *et al*., 2017;).

To transiently express NS1 in *N. benthamiana*, the *A. tumefaciens* strains carrying pMYDV1 or pMYDV2, together with *A. tumefaciens* carrying pMYV508, were inoculated into 5 mL of liquid LB

medium containing Kan and rifampicin (50 µg/mL each) and co-cultured for 2 days at 28 ºC. Cells were harvested and resuspended in MES infiltration buffer (10 mM MES, 10 mM $MgSO_4$, pH 5.5, 200 µM acetosyringone) to an optical density at 600 nm of 0.8. Agroinfiltration was carried out on 6-week-old *N. benthamiana* leaves using 1 ml needle-free syringes. The infiltrated leaves were harvested after 3, 5, and 7 days after infiltration (3, 5, and 7 day-post-infiltration, dpi). *N. benthamina* leaves from the same plants not being infiltrated were used as a negative control.

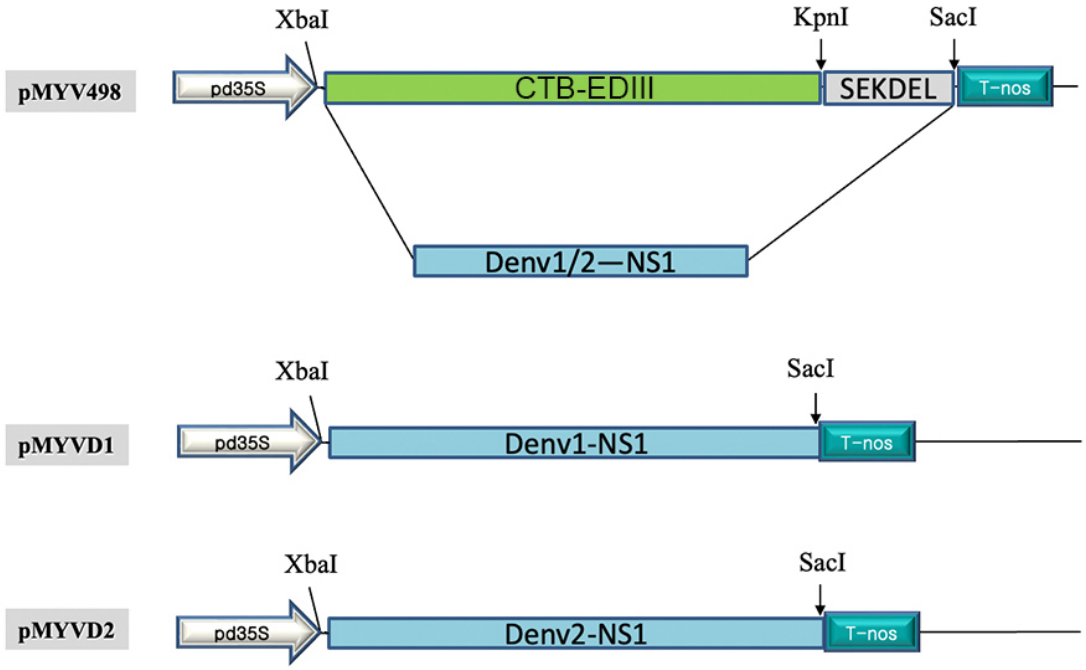


Figure 1. Map of the expression constructs of *Denv1-NS1* and *Denv2-NS1*, which were cloned into pMYV498 to create two new recombinant vectors: pMYVD1 and pMYVD2, respectively. The use of *Sac*I instead of *Kpn*I eliminated SEKDEL, the endoplasmic reticulum (ER) retention signal.

Protein extraction and Western blot

The infiltrated and non-infiltrated leaves were harvested at 3, 5, and 7 dpi. Total soluble protein was extracted from leaf tissue by grinding it in liquid nitrogen using a mortar and pestle. The homogenized tissue was resuspended in PBS (NaCl: 137 mM, KCl: 2.7 mM, Na_2HPO_4: 10mM, and KH_2PO_4: 1.8 mM) buffer pH 7.4, and cell debris was separated from the protein extract by centrifugation at 13,500 rpm for 15 minutes at 4 ºC. The concentration of protein samples was determined by a Bradford assay.

The protein extract samples were subjected to Western blot analysis. Due to a lack of specific antibodies against consensus NS1, we used sera from dengue patients who were admitted to the hospital at Tay Nguyen General Hospital during the period from September to December 2022 as the primary antibody. These samples were part of routine epidemiological surveys by the Tay Nguyen Institute of Hygiene and Epidemiology and had been stored at the institute; therefore, approval by a bioethics committee was not required. Before use, the sera were tested

again using the Duo Dengue Ag-IgG/IgM Rapid Test (CTK Biotech). Banked sera tested negative were used as a negative control.

Western blot analysis was carried out as described before (Nguyen *et al*., 2022) with some modifications. Protein samples were analyzed in denaturing (boiling for 10 minutes before loading) and non-denaturing conditions (no boiling prior to loading) on SDS-PAGE gel. For the denaturing condition, approximately 30 µg of protein extract were analyzed on each well, while for non-denaturing condition, approximately 50 µg of protein extract were analyzed on each well. Patients' sera were diluted at 1:500 in blocking buffer and used as the primary antibody. AP-conjugated rabbit anti-human IgG and anti-human IgM (Benthyl Cat. No. A80-119AP and A80-101AP, respectively) were used as the secondary antibodies, at a 1:5000 dilution. *E. coli* expressed and purified DENV1-NS1 and DENV2-NS1 were used as positive controls while the total protein extract from non-infiltrated leaves was used as a negative control.

Dot blot analysis of *N. benthamiana*-expressed NS1

All protein samples were mixed with 1% SDS and adjusted to equimolar concentrations. Approximately 5 µg of *N. benthamiana* leaf protein extract (equivalent to 2–2.5 µL) was blotted on a nitrocellulose membrane on a dot of approximately 1–1.5 cm in diameter. For a positive control, a mixture of 100 ng of *E. coli* expressed and purified DENV1-NS1 and DENV2-NS1 was used. The membranes were allowed to dry overnight at room temperature (approximately 8–10 hours). The following day, the membranes were washed three times with TBST buffer (Tris/HCl pH7.5: 20 mM, NaCl: 150 mM, and Tween® 20: 0.1%), each time for 5 minutes. Subsequently, the membranes were blocked by being submerged in 5% skim milk prepared with TBST for 30 minutes. Dengue patients' antisera were used as the primary antibody (1:500 dilution). The membranes were incubated with the primary antibodies for 30 minutes, followed by 3 x 5 minute washes with TBST. After the last wash, the membranes were incubated with the alkaline phosphatase-conjugated secondary antibody (Rabbit anti-human IgG; see above for details) for 30 minutes. Following a 3 x 5 minute wash, the membrane was re-balanced in TMN buffer (NaCl: 100 mM, $MgCl_2$: 50 mM, and Tris/HCl pH9.5: 100 mM) for 5 minutes before being developed with an NBT/BCIP solution.

RESULTS AND DISCUSSION

Acquisition of the global consensus DENV1-NS1 and DENV2-NS1

At the time of analysis (June 2020), we obtained 1726 polyprotein sequences (3341–3392 aa) from dengue virus serotype 1 and 1415 polyprotein sequences (3391 aa) from dengue virus type 2 for consensus sequence identification. The consensus sequences for DENV1-NS1 and DENV2-NS1 were identified as AYP74685.1 and AFK65750.1, respectively. Since the meaning of consensus sequence here indicates the most frequently encountered variant in the database, there must be a veritable multitude of Dengue variants whose sequences are identical to the above sequences. Therefore, the accession numbers above were provided for the sake of reference and searchability.

To double-check that these sequences are indeed global consensus representative

sequences, we searched the NCBI non-redundant protein database using the above sequences as queries. For Dengue serotype 1, the number of entries with a 100% sequence match is 198, while the number of entries with just one mismatch is over 500. For Dengue serotype 2, the number of entries with a 100% sequence match is over 500. Given that the total number of polyprotein and NS1 sequences of both viruses stand at around four to five thousand entries (searching Entrez using the keywords Dengue virus type 1/2 polyprotein or NS1 on 10/10/2023), these results indicate that indeed we have obtained the global consensus sequences of NS1 for Dengue virus types 1 and 2.

Considering the sequence heterogeneity within each canonical serotype as well as potential artefacts like sequencing and sequence submission errors, our approach to generating the global consensus sequences for NS1 serves to mitigate unnecessary errors and at the same time, ensuring that the applicability of the antigens is maximized and any potential discrepancies are minimized.

Our approach is much simpler than that of Park *et al.* (2020), who also aimed to create consensus NS1 for all flaviviruses using reclassification based on epitope phylogenetic relationships. This requires expert knowledge on immunology and the phylogenetic relationship of dengue viruses, which is, therefore, probably beyond the capability of many researchers. Furthermore, the resulted consensus proteins need to go through extensive experimental verification to ensure that the structure and immunogenicity are not altered, due to the lack of such proteins in nature.

We also used Pepripred 2.0 (available at https://www.iedb.org) to predict linear epitopes on DENV1-NS1 and DENV2-NS1. Subsequently, we searched the epitope database (https://www.iedb.org) to match the predicted epitopes with experimentally verified ones. The results are presented in Supplementary Figure 1. Most verified epitopes either match completely with our consensus NS1 or have only one to two mismatches over the length of the epitopes. This adds further credibility to our consensus NS1 sequences.

Cloning of *Denv1-NS1* and *Denv2-NS1* into pMYV498

From a list of codon-optimized genes for *N. benthamiana* expression, we selected two genes encoding for the global consensus DENV1-NS1 and DENV2-NS1 (designated as *Denv1-NS1* and *Denv2-NS1*, GenBank accession numbers OR349280 and OR349282, respectively) based on their CAIs, mRNA folding energy, and percentage of 3rd position GC content. High CAI helps avoid rare codon problems, while adequate mRNA folding energy allows for a long mRNA half-life while facilitating ribosome entry, and high 3rd position GC content is a common feature of regions containing protein-coding genes (Jung, McDonal, 2011). Codon optimization is an essential step for adequate protein expression in plants (Webster *et al*., 2017). The genes were ordered at PhusaGenomics company with *Xba*I and *Sac*I restriction enzyme sequences, respectively, were added at the 5' and 3' termini for ease of directional cloning into pMYV498.

The sequences of each of the two genes were digested by *Xba*I/*Sac*I, released from pUC19, and cloned into pMYV498 pre-

linearized with the same pair of enzymes. The resulting plasmids are named pMYDV1 and pMYDV2, respectively, and have the size of approximately 11 kb (Fig. 2).

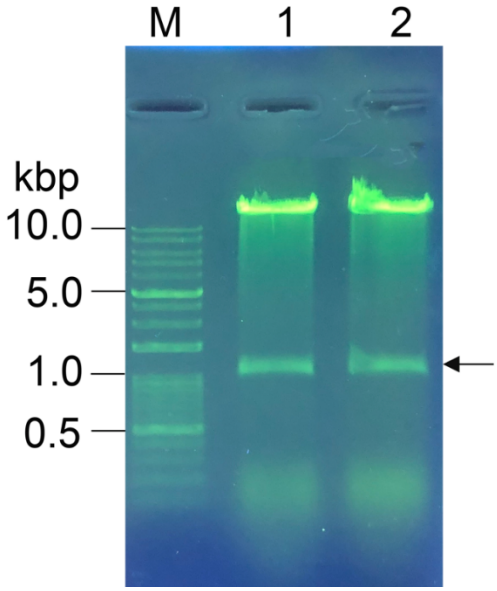


Figure 2. Analysis of recombinant pMYVD1 and pMYVD2 by restriction digestion. The recombinant vectors pMYVD1 and pMYVD2 were digested with *Xba*I/*Sac*I and analyzed on a 1% agarose gel. The arrow indicates the position of *Denv1-NS1* and *Denv2-NS1* genes.

Expression analysis of DENV1-NS1 and DENV2-NS1

In our investigation of NS1 antigen expression, we utilized human polyclonal antibodies as a detection method. The choice is based on the constraint of lacking specific antibodies from other species against the NS1, combined with the convenient availability of human sera. The human sera were stored for previous retrospective seroepidemiological investigations, thus obviated the need for additional bioethics approval. The choice is also more appropriate in this context since it also provided a more clinically relevant measure of antigenic recognition.

Based on the Western blot using Dengue patients' sera diluted at 1:500 as the primary antibodies and AP-conjugated, rabbit anti-human IgG as the secondary antibody, we could see that DENV1-NS1 and DENV2-NS1 were successfully expressed as approximately 40 kDa proteins under denaturing conditions (Fig. 3). There was variation among leaves harvested at different intervals, with 5 dpi and 7 dpi samples consistently showing higher expression levels than 3 dpi samples. Due to the absence of specific antibodies, our conclusion is rather provisional at best. Nguyen *et al.* (2016) reported somewhat similar results, where protein expression reached the highest level at 4 to 6 dpi. Since NS1 could assemble into multimeric forms (Glasner *et al*., 2018), we tested whether our recombinant NS1s could assemble into higher multimeric configurations. To do this, we repeated the Western blot but used the protein extract under non-boiling (non-denaturing)

condition. The result is presented in Figure 4. As seen in Fig. 4, the protein extract from agroinfiltrated leaves showed smearing signals above 40 kDa, while those were absent in the negative control sample. Since the Western blot using rabbit anti-human IgM failed to detect any signal (data not shown), it was excluded from further analyses.

To ensure that the Western blot signals are authentic instead of artefacts, we used sera tested negative with the Dengue Duo quick test as the primary antibody in a Western blot analysis where *N. benthamiana* expressed NS1 were analyzed under denaturing and non-denaturing conditions. The result is shown in Supplementary Figure 2. Contrary to the sera from Dengue-positive patients, there was no signal indicating that there was interaction between specific antibodies from the sera and the antigens used in the Western blot.

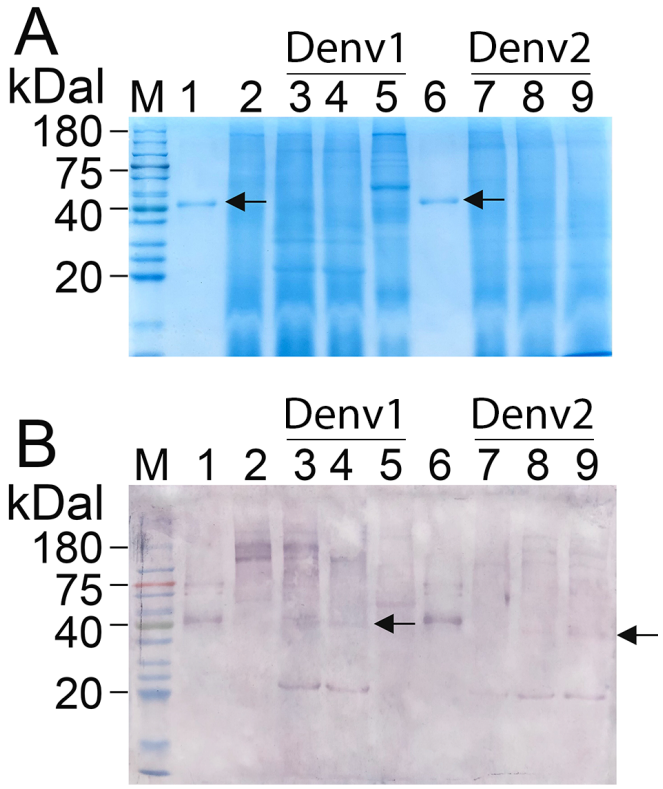


Figure 3. Western blot analysis of the protein extract from *N. benthamiana* leaves under denaturing (boiling) condition. M: prestained protein ladder (Smobio 3 PM5100). Lane 1 and lane 6 contain 100 ng of *E. coli* expressed and purified NS1 from DENV1 and DENV2, respectively, as positive controls. Lane 2: protein extract from non-infiltrated *N. benthamiana* leaves as negative control. Lanes 3, 4 and 5 contain 30 µg protein extract from 3, 5 and 7 dpi *N. benthamiana* leaves expressing DENV1-NS1; lanes 7, 8 and 9 contain 30 µg protein extract from 3, 5 and 7 dpi *N. benthamiana* leaves expressing DENV2-NS1, respectively. A: 12% SDS-PAGE image and B: Western blot result from the twin gel of A using a Dengue patient serum (1:500 dilution) as the primary antibody and AP-conjugated, rabbit anti-human IgG as the secondary antibody. The arrows showed the expected positions of monomeric DENV1-NS1 and DENV2-NS1, which have a predicted molecular weight of 40 kDa.

Dengue NS1s have been extensively used as the main antigen to detect specific antibodies in patients' sera (Kok *et al*., 2023). Therefore, to facilitate the production of NS1-based diagnostic products, recombinant NS1 has been expressed in several hosts. The most common is *E. coli*. However, NS1 proteins were usually expressed in insoluble form and needed refolding. Upon refolding, only a small fraction of the recombinant NS1 usually forms dimers (Das *et al*., 2009; Allonso *et al*., 2011), the main conformation of NS1 at the endoplasmic reticulum (ER) in the host cell (Glasner *et al*., 2018). Recombinant Dengue NS1 has also been produced in *N. benthamiana* (Marques *et al*. 2020), and the authors showed that NS1 formed higher conformational structures such as dimer, trimer, and tetramer. This is indeed what we saw in the Western blot analysis of our consensus NS1 in non-denaturing form, showing the advantages of using *N. benthamiana* over *E. coli* for recombinant NS1 production.

One of the main limits of our study is the lack of specific antibodies against NS1. In addition, we have not made use of the consensus Kozak sequence for *N. benthamiana*, which was shown to improve the expression of target proteins significantly (Kanagarajan *et al*., 2012; Kim *et al*., 2014). Furthermore, the use of *Sac*I restriction enzyme instead of *Kpn*I (see Fig. 1) eliminated the ER retention signal, which helps retain the expressed protein in ER instead of transporting it to the Golgi apparatus, where plant glycosylation may occur. Plant glycosylation may have a negative effect on the immunogenicity and activity of plant-expressed recombinant proteins (Shoji *et al*., 2008). It should be noted that to make use of the SEKDEL signal, the target protein needs a leading sequence. In future research, we aim to redesign *Denv1-NS1* and *Denv2-NS1* to include a purification and detection epitope such as His-Tag, a secretory signal peptide, and make use of the RE retention signal.

Dot-blot using *N. benthamiana*-expressed NS1

To test whether *N. benthamiana* expressed DENV1-NS1 and DENV2-NS1 could find practical application, we tried the dot-blot method. Dot-blot is similar to Western blot but faster, cheaper, and more convenient. Indeed, dot-blot had been tested in acute Dengue patients and showed robustness compared with standard tests such as RT-PCR or quick-test (Koraka *et al*., 2003). The dot-blot results are shown in Figure 5. There were some variations in the IgG titers among serum samples, as some sera showed stronger differential signals between proteins extract from agroinfiltrated leaves and non-infiltrated leaves. Nevertheless, the signals seem to be sufficient to differentiate between negative and positive Dengue sera. It is interesting to observe that the Western blot and dot blot setup in this study seemed to be more sensitive than the Onsite Duo Dengue Ag-IgG/IgM Rapid Test (Supplementary Figure 3). The main limit is that in unpurified form, noises prevent differential diagnosis between dengue serotypes. Furthermore, a previous study also showed that dot blot could be more sensitive than a quick test (Koraka *et al*., 2003) in detecting dengue in acute patients. With appropriate automation, dot blot can be an extremely versatile and high-throughput diagnostic tool for dengue diagnosis, capable of differentiating serotypes (Auerswald *et al*., 2019).

Since the sera are from acute dengue patients, it is natural to see a higher IgG titer than an IgM titer (Kok *et al*. 2023). Due to our limited access to clinical samples, the serum samples used in this study are probably not ideal for the IgG/IgM capturing assay, as the quick test results could not show a positive signal for IgM/IgG, despite showing a positive signal for the NS1 antigen.

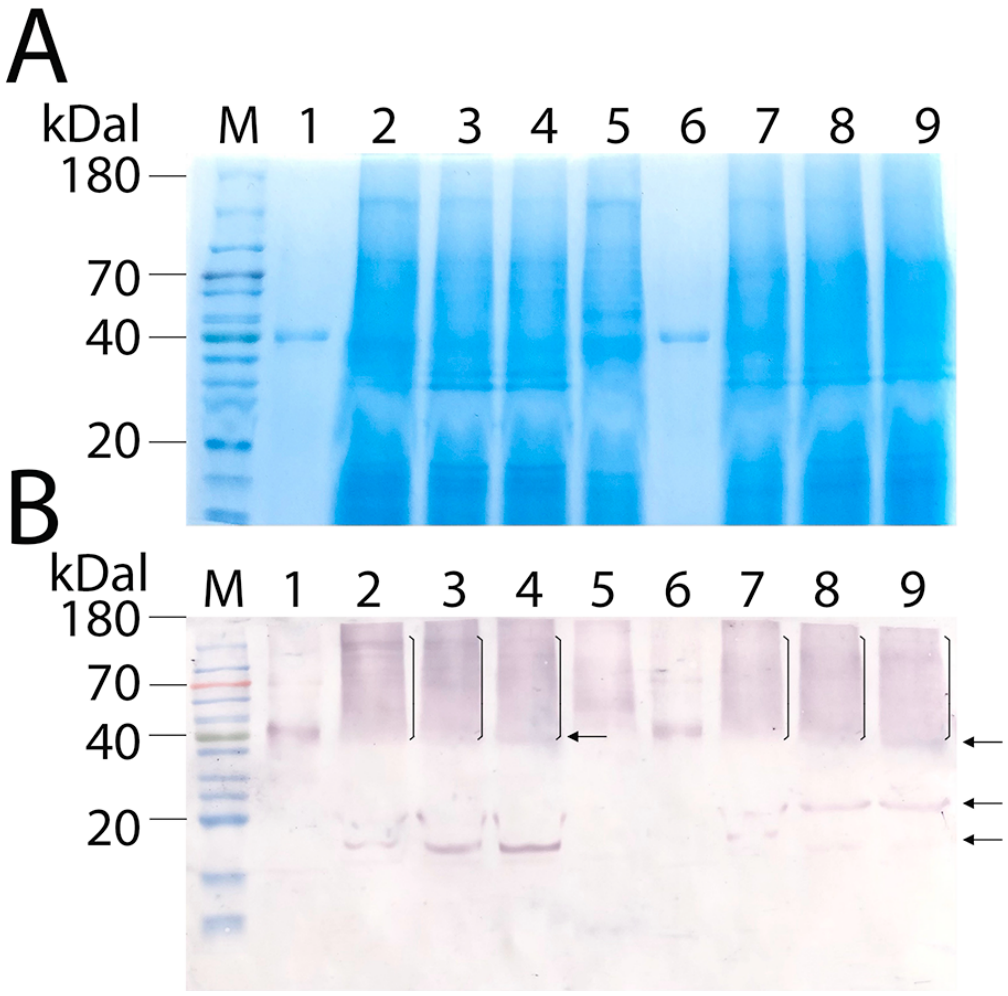


Figure 4. Western blot analysis of the protein extract from *N. benthamiana* leaves under non-denaturing (non-boiling) condition. Lane M: prestained protein ladder (Smobio 3 PM5100). Lane 1 and lane 6 contain 100 ng of *E. coli* expressed and purified NS1 from DENV1 and DENV2, respectively, for positive control. Lane 5 contains 50 µg of protein extract from non-infiltrated *N. benthamiana* leaves, which was used as a negative control. Lanes 2, 3, 4 and 7, 8, and 9 contain 50 µg of protein extract from 3, 5, and 7 dpi *N. benthamiana* leaves expressing DENV1-NS1 and DENV2-NS, respectively. A: 12% SDS-PAGE image and B: Western blot result from the twin gel of A using a Dengue patient serum (1:500 dilution) as the primary antibody and AP-conjugated, rabbit anti-human IgG as the secondary antibody. The arrows show the positions of bands that appeared in the test samples but were absent from the negative samples. The brackets showed supposedly multimeric forms of NS1 proteins.

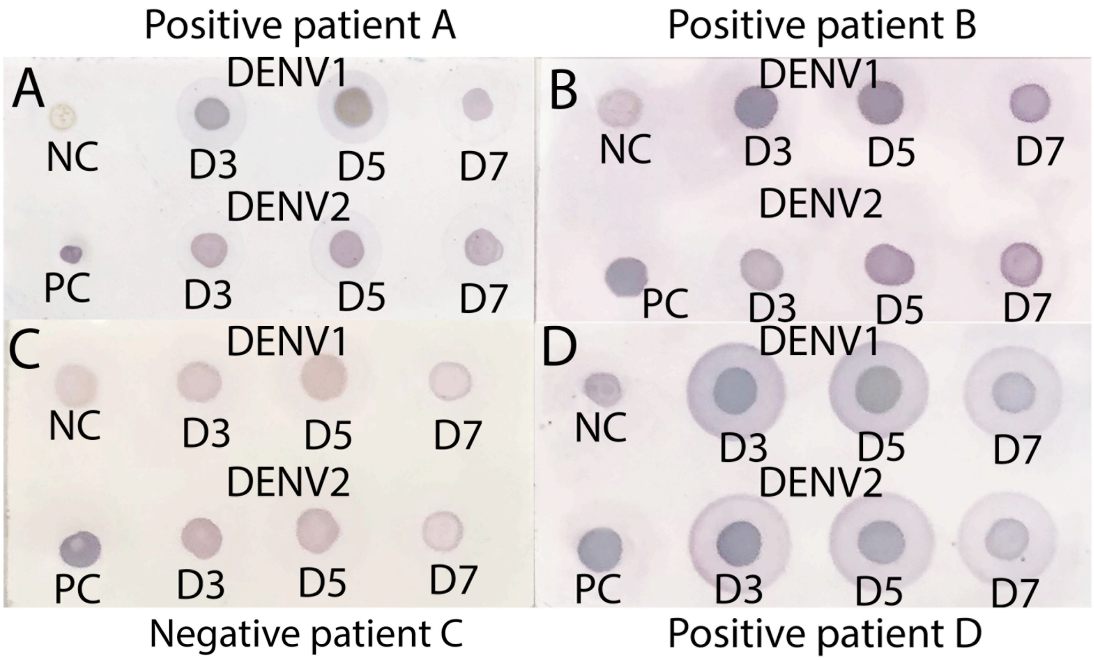


Figure 5. Dot-blot analysis using *N. benthaminana* protein extract as capturing antigen. A, B, and D are dot-blot results from three different acute dengue patients' sera, while C is the result from a dengue negative patient's serum. NC contains protein extract from non-infiltrated *N. benthamiana* leaves.

CONCLUSION

We reported an initial finding of expressing global consensus non-structural protein (NS1) of dengue virus types 1 and 2 in *N. benthamiana*. The consensus NS1 sequences were shown to be the most commonly encountered variants based on BLAST searches. The *N. benthamiana* codon-optimized genes encoding for these consensus NS1 were successfully expressed by agroinfiltration, as shown by Western blot using acute dengue patients' sera. However, the expression level could still be improved through redesigning the expression construct. In its unpurified form, *N. benthamiana*-expressed NS1 showed some promising applicability for dengue diagnosis such as Western or dot blot.

Acknowledgement: *This study is supported by the Grant: "Production of recombinant non-structural protein 1 of Dengue and Zika viruses.". We would like to thank Dak Lak's Department of Science and Technology for supporting this work.*

REFERENCES

Auerswald H, Klepsch L, Schreiber S, Hülsemann J, Franzke K, Kann S, Y B, Duong V, Buchy P, Schreiber M (2019) The dengue ED3 dot assay, a novel serological test for the detection of dengue virus type-specific sntibodies and its application in a retrospective seroprevalence study. *Viruses* 11: 304.

Allonso D, da Silva Rosa M, Coelho DR, da Costa SM, Nogueira RM, Bozza FA, Santos FB, de Barcelos Alves AM, Mohana-Borges R (2011) Polyclonal antibodies against properly folded Dengue virus NS1 protein expressed in *E. coli*

enable sensitive and early dengue diagnosis. *J. Virol Methods* **175**: 109–116.

Bally J, Jung H, Mortimer C, Naim F, Philips JG, Hellens R, Bombarely A, Goodin MM, Waterhouse PM (2018) The rise and rise of *Nicotiana benthamiana*: A plant for pll reasons. *Annu Rev Phytopathol* 56: 405–426.

Cattarino L, Rodriguez-Barraquer I, Imai N, Cummings DAT, Ferguson NM (2020) Mapping global variation in dengue transmission intensity. *Sci Transl Med* 12: eaax4144.

Danecek P, Lu W, Schein CH (2010) PCP consensus sequences of flaviviruses: correlating variance with vector competence and disease phenotype. *J Mol Biol* 396: 550–563.

Das D, Mongkolaungkoon S, Suresh MR (2009) Super induction of dengue virus NS1 protein in *E. coli*. *Protein Expr Purif* 66: 66–72.

Edgar RC (2004) MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* 5: 113.

Foucambert P, Esbrand FD, Zafar S, Panthangi V, Cyril Kurupp AR, Raju A, Luthra G, Shahbaz M, Almatooq H, Khan S (2022) Efficacy of dengue vaccines in the prevention of severe dengue in children: A systematic review. *Cureus* 14: e28916.

Glasner DR, Puerta-Guardo H, Beatty PR, Harris E (2018) The good, the bad, and the shocking: The multiple roles of dengue virus nonstructural protein 1 in protection and pathogenesis. *Annu Rev Virol* 5: 227–253.

Guzman MG, Halstead SB, Artsob H, Buchy P, Farrar J, Gubler DJ, Hunsperger E, Kroeger A, Margolis HS, Martínez E *et al*. (2010) Dengue: a continuing global threat. *Nat Rev Microbiol* 8: S7–16.

Horsch RB, Fry JE, Hoffmann NL, Wallfroth M, Eichholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. *Science* 227: 1229–1231.

Hussain M, Idrees M, Afzal S (2015) Development of global consensus of dengue virus envelope glycoprotein for epitopes based vaccine design. *Curr Comput Aided Drug Des* 11: 84–97.

Jung SK, McDonald K (2012) Visual gene developer: a fully programmable bioinformatics software for synthetic gene optimization. *BMC Bioinformatics* 12: 340.

Kanagarajan S, Muthusamy S, Gliszczyńska A, Lundgren A, Brodelius PE (2012) Functional expression and characterization of sesquiterpene synthases from *Artemisia annua* L. using transient expression system in *Nicotiana benthamiana*. *Plant Cell Rep* 31: 1309–1319.

Katzelnick LC, Fonville JM, Gromowski GD, Bustos Arriaga J, Green A, James SL, Lau L, Montoya M, Wang C, VanBlargan LA *et al*. (2015) Dengue viruses cluster antigenically but not as discrete serotypes. *Science* 349: 1338–1343.

Katzelnick LC, Gresh L, Halloran ME, Mercado JC, Kuan G, Gordon A, Balmaseda A, Harris E (2017) Antibody-dependent enhancement of severe dengue disease in humans. *Science* 358: 929–932.

Katzelnick LC, Coello Escoto A, Huang AT, Garcia-Carreras B, Chowdhury N, Maljkovic Berry I, Chavez C, Buchy P, Duong V *et al*. (2021) Antigenic evolution of dengue viruses over 20 years. *Science* 374: 999–1004.

Kim Y, Lee G, Jeon E, Sohn EJ, Lee Y, Kang H, Lee DW, Kim DH, Hwang I (2014) The immediate upstream region of the 5'-UTR from the AUG start codon has a pronounced effect on the translational efficiency in *Arabidopsis thaliana*. *Nucl Acids Res* 42(1): 485–498.

Kok BH, Lim HT, Lim CP, Lai NS, Leow CY, Lew CH (2023) Dengue virus infection – a review of pathogenesis, vaccines, diagnosis and therapy. *Virus Res* 324: 199018.

Koraka P, Burghoorn-Maas CP, Falconar A, Setiati TE, Djamiatun K, Groen J, Osterhaus AD (2003) Detection of immune-complex-dissociated nonstructural-1 antigen in patients

with acute dengue virus infections. *J Clin Microbiol* 41: 4154–4159.

Leng CH, Liu SJ, Tsai JP, Li YS, Chen MY, Liu HH, Lien SP, Yueh A, Hsiao KN, Lai LW *et al*. (2009) A novel dengue vaccine candidate that induces cross-neutralizing antibodies and memory immunity. *Microbes Infect* 11: 288–295.

Marques LÉC, Silva BB, Dutra RF, Florean EOPT, Menassa R, Guedes MIF (2020) Transient Expression of Dengue Virus NS1 Antigen in *Nicotiana benthamiana* for Use as a Diagnostic Antigen. *Front Plant Sci* 10: 1674.

Nguyen TT, Lundkvist Å, Lindahl J (2019) Urban transmission of mosquito-borne flaviviruses - a review of the risk for humans in Vietnam. *Infect Ecol Epidemiol* 9: 1660129.

Nguyen XH, Kim MY (2017) Overexpression and oral immunogenicity of a dengue antigen transiently expressed in *Nicotiana benthamiana*. *Plant Cell Tissue Organ Cult* 131: 567–577.

Nguyen XH, Nguyen QDT, Kim MY, Kim TG, Jang YS, Yang MS (2016) Immunogenicity of an S1D epitope from porcine epidemic diarrhea virus and cholera toxin B subunit fusion protein transiently expressed in infiltrated *Nicotiana benthamiana* leaves. *Plant Cell Tissue Organ Cult* 127: 369–380.

Pang T, Cardosa MJ, Guzman MG (2007) Of cascades and perfect storms: the immunopathogenesis of dengue haemorrhagic fever-dengue shock syndrome (DHF/DSS). *Immunol Cell Biol* 85: 43–45.

Park C, Kim WB, Cho SY, Oh EJ, Lee H, Kang K, Lee Y, Lee DG (2020) A Simple Method for the Design and Development of Flavivirus NS1 Recombinant Proteins Using an In Silico Approach. *Biomed Res Int* 2020: 3865707.

Peyret H, Lomonossoff GP (2015) When plant virology met Agrobacterium: the rise of the deconstructed clones. *Plant Biotechnol J* 3:1121–1135.

Pierson TC, Diamond MS (2013) Flaviviruses, pp. 757–758. In Knipe DM and Howley PM (eds), Fields Virology Vol 1 6th Ed. Lippincott Williams & Wilkins, Philadelphia, PA.

WHO (2022) Dengue and severe dengue, accessed at: (https://www.whoint/news-room/fact-sheets/detail/dengue-and-severe-dengue).

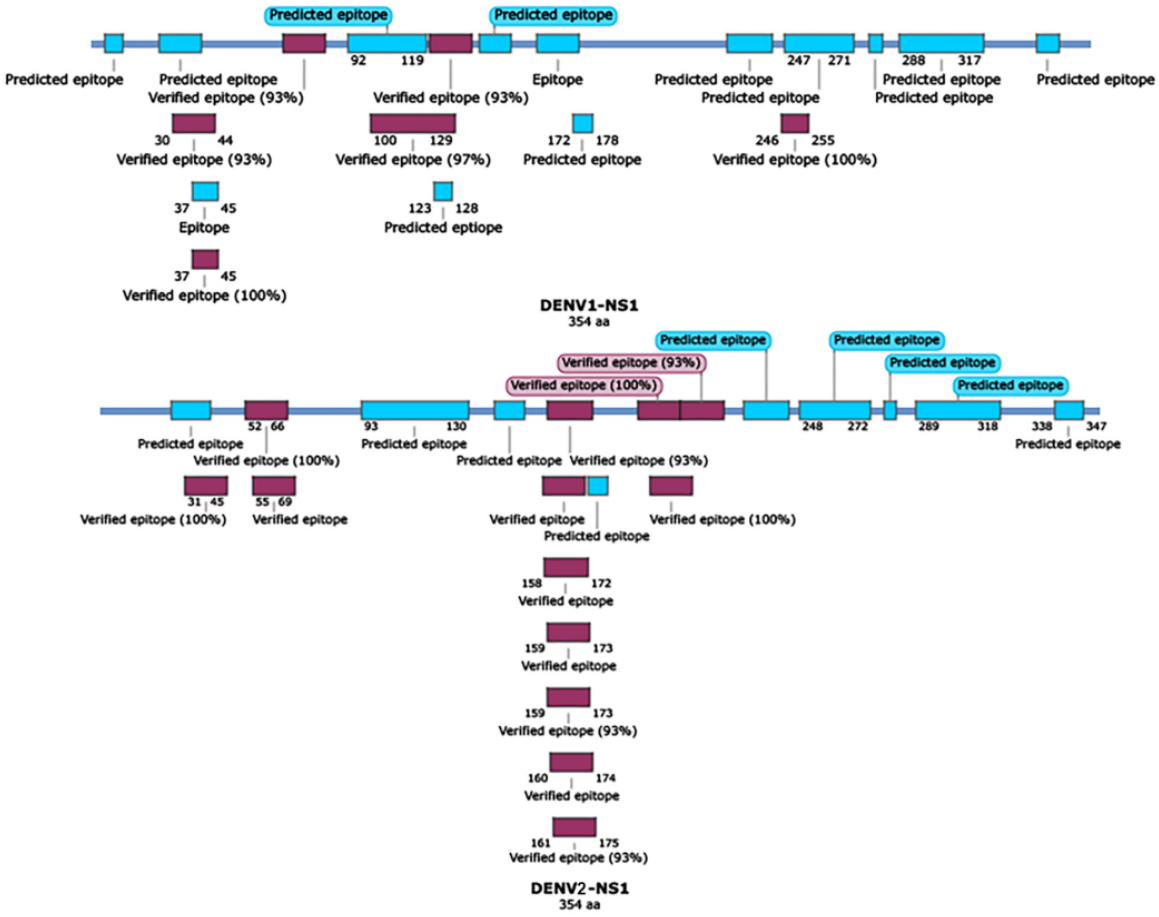
Sankaradoss A, Jagtap S, Nazir J, Moula SE, Modak A, Fialho J, Iyer M, Shastri JS, Dias M, Gadepalli R *et al*. (2022) Immune profile and responses of a novel dengue DNA vaccine encoding an EDIII-NS1 consensus design based on Indo-African sequences. *Mol Ther* 30:2058–2077.

Sarker A, Dhama N, Gupta RD (2023) Dengue virus neutralizing antibody: a review of targets, cross-reactivity, and antibody-dependent enhancement. *Front Immunol* 14: 1200195.

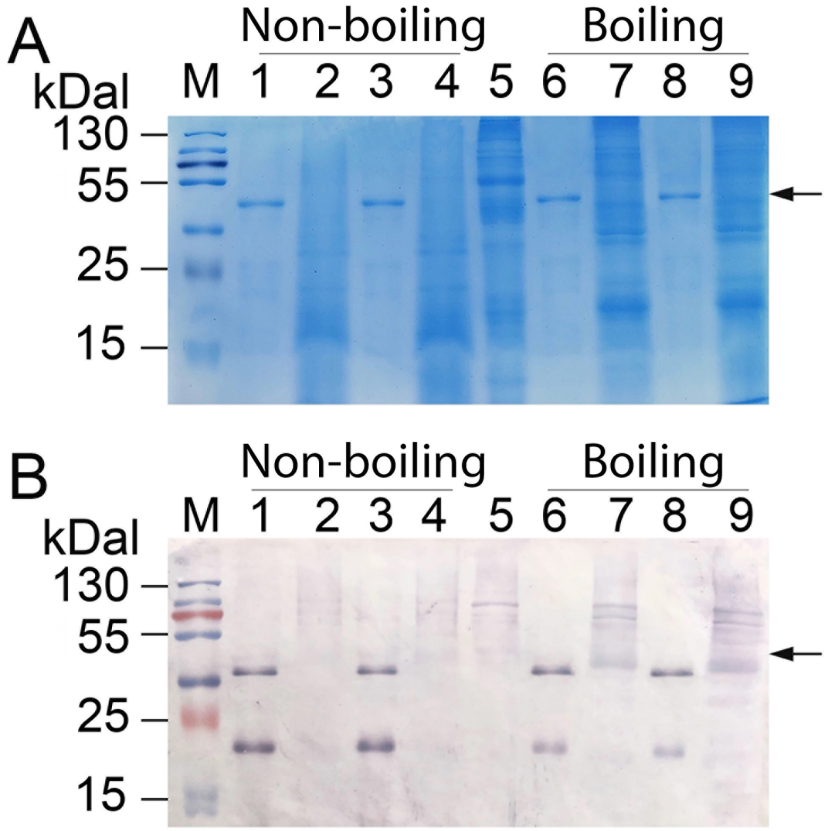
Shoji Y, Chichester JA, Bi H, Musiychuk K, de la Rosa P, Goldschmidt L, Horsey A, Ugulava N, Palmer GA, Mett V, Yusibov V (2008) Plant-expressed HA as a seasonal influenza vaccine candidate. *Vaccine* 26: 2930–2934.

Tamura K, Stecher G, Kumar S (2021) MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Mol Biol Evol* 38: 3022–3027.

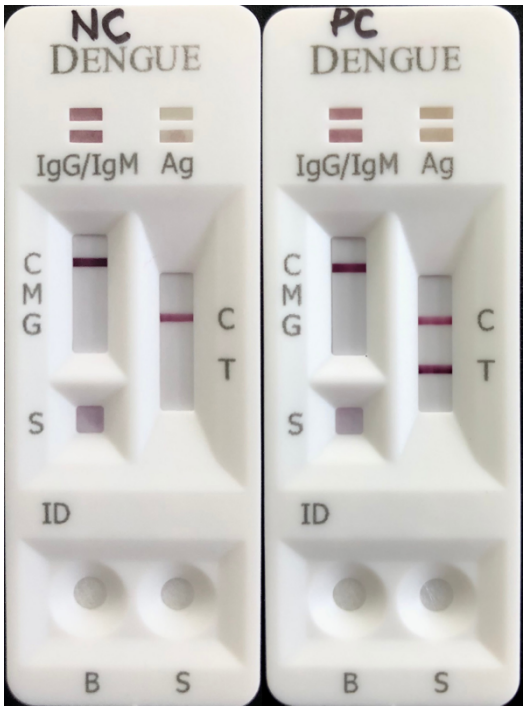
Webster GR, Teh AY, Ma JK (2017) Synthetic gene design-The rationale for codon optimization and implications for molecular pharming in plants. *Biotechnol Bioeng* 114: 492–502.



Supplementary Figure 1. Mapping and matching predicted linear epitopes and experimentally verified linear epitopes on DENV1-NS1 and DENV2-NS1.



Supplementary Figure 2. Western blot analysis using dengue negative patient serum (1:500 dilution) as the primary antibody and AP-conjugated, rabbit anti-human IgG as the secondary antibody. M: prestained protein ladder (Thermofisher 26616). Lane 1, 6, and 2, 7 contain 100 ng of *E. coli* expressed and purified DENV1-NS1 and DENV2-NS1, respectively, as positive controls; lane 2 and 7 contain 30 µg of 5 dpi *N. benthaminana* leaf protein extract expressing DENV1-NS1; lane 4 and 9 contain 30 µg of 5 dpi *N. benthamiana* leaf protein extract expressing DENV2-NS1; and lane 6 contains 30 µg of non-infiltrated *N. benthamiana* leaf protein extract. The arrow shows the expected position of NS1, which is approximately 40 kDa.



Supplementary Figure 3. A representative result of the Ag-IgG/IgM Rapid Test (CTK Biotech) showing positive and negative result of the patients' sera. Sera were loaded, and the results were read after 15 minutes, according to the instruction manual. Positive dengue patients showed positive results in the NS1 antigen test but negative results in the IgM/IgG capture test, while negative patients showed negative results in both tests.