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Generation of dengue 3 envelope domain III using tobacco mosaic virus-based vector system and its immunological response mouse model by generating anti-dengue virus antibodies

Hailah M. Almohaimeed[1*], Rasha Assiri[2], Waheeb S. Aggad[3]

[1] *Department of Basic Science, College of Medicine, Princess Nourah bint Abdulrahman University, P. O. Box 84428, Riyadh 11671, Saudi Arabia*

[2] *Department of Basic Medical Sciences, College of Medicine, Princess Nourah Bint Abdulrahman, University, Riyadh, P.O.Box 11671, Saudi Arabia*

[3] *Division of Anatomy, Department of Basic Medical Sciences, College of Medicine, University of Jeddah, Jeddah 23890, Saudi Arabia*

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Abstract

Producing recombinant proteins in plants has become a valuable alternative to traditional microbial or mammalian systems due to its cost-effectiveness, scalability, and ability to perform post-translational modifications. This study investigates the use of the Tobacco Mosaic Virus (TMV)-based vector system for producing the Dengue virus serotype 3 (DENV-3) envelope domain III (EDIII) protein in plants.. A fragment of the gene that encodes domain III of the dengue 3 envelope protein (D3EIII, comprising 300-420 amino acids), was effectively expressed within *Nicotiana tabacum* plants utilizing a transient expression system based on tobacco mosaic virus (TMV). The N-terminal 5' UTR region upstream of D3EIII notably enhanced protein yield in infected tissues. The produced recombinant protein exhibited reactivity with both (anti) D3EIII polyclonal antibodies and antibodies of anti-His tag. Upon injection of EDIII in mice, it stimulated the generation of antibodies against the dengue-specific virus. The induced antibodies demonstrated neutralizing activity against dengue virus type 3. These findings indicate that the TMV expression system is effective for producing dengue virus antigens in plants, resulting in antigens with appropriate properties and strong immunogenic potential.

Keywords: Recombinant proteins; Tobacco Mosaic Virus (TMV); Dengue virus; Plant-based expression system; Immunogenic potential

1. Introduction

Dengue fever, caused by the Dengue virus, is a substantial communal health concern in tropical and subtropical regions worldwide. The Dengue virus has 4 distinct serotypes (DENV-1-4), and infection with single serotype does not deliver immunity alongside the others. Vaccines against Dengue have been developed, but their efficacy varies among different serotypes. The envelope (E) protein of the Dengue virus plays a crucial role in virus attachment and entry into host cells. Domain III of the Dengue E protein (EDIII) contains epitopes that elicit neutralizing antibodies, making it an attractive target for vaccine development. Among the array of plant viral vectors explored thus far, utilizing a TMV-based expression system emerges as a favorable avenue for promptly stating biologically active proteins. This approach holds the potential to bypass the time constraints associated with utilizing steady transgenic plants [1]. Numerous therapeutic proteins of humanoid and animal origin have been effectively expressed using recombinant TMV. This has been achieved through different methods, including read-through fusions to a coat protein (CP) [2, 3], direct-fusion with a CP [4, 5], and enclosure under the ruling of CP sub-genomic promoters [6-11]. However, the initial two methods were limited to expressing short peptides approximately 7–25 amino acids (aa) in length [12], the latest approach represents a breakthrough, enabling the production of bigger recombinant proteins (130 kDa), i.e. the 16 L1 protein of the human papillomavirus [9].

The predominant focus of recombinant DNA strategies for production of vaccines has centered on the envelope protein of the dengue. With a length of 495 amino acids (aa), the E glycoprotein serves as the principal structural component prominently displayed on the external of matured dengue virions. It plays an important role in the connection and entrance into host cells and serves as the

* Corresponding author.
E-mail address: halamodm1444@gmail.com, hmalmohaimeed@pnu.edu.sa (H. M. Almohaimeed).
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primary antigen responsible for inducing protective immunity and subsequent virus neutralization [13]. Structurally, the E glycoprotein comprises three discernible domains: I, II, and III [14]. Despite its significance, expressing this E glycoprotein in various multiple differential, such as E. coli [15], P. pastoris [16, 17], and baculovirus [18], has resulted in substantial produces of the immunogenic and antigenic dengue 3 enevlope protein. Nonetheless, challenges persist, including low expression levels of the long or ecto-domain of the dengue 3E protein and susceptibility to protein degradation [15]. Consequently, researchers have investigated the use of smaller fragments encompassing the biologically crucial domain III of the envelope protein. Studies have shown that chimeric proteins containing the dengue virus serotype 3 envelope domain III (D3EIII) exhibit significant immunogenic properties, capable of eliciting neutralizing antibodies in experimental animal models. [19].

In this study, we used a TMV-based vector, derived from the cross-tomato mosaic virus (ToMV) [12], to produce the D3EIII protein in tobacco plants. Additionally, we optimized the construct to improve expression levels by incorporating the TMV untranslated region (UTR) and a signal peptide into the D3EIII sequence. Subsequently, the new protein was synthesized, refined, and administered for mouse immunization. The resultant recombinant D3EIII, synthesized in tobacco plants, prompted the generation of anti-dengue virus antibodies possessing counteracting capabilities.

2. Materials and Methods

2.1. Construction of the expression system

The expression of the D3EIII protein in tobacco involved the creation of two distinct manifestation vectors based on TocJ [12]. The first vector, termed pSP/D3EIII/SEKDEL/His6, comprised D3EIII along with the N-terminal peptide signal, the C-terminal endoplasmic ER holding sequence specific to plants and a 6 consecutive histidine sequence named as (His6) tag. The second vector, denoted pδ/SP/D3EIII/SEKDEL/His6, featured the inclusion of the 5' UTR-δ upstream enhance element of signal peptide, along with the components present in pSP/D3EIII/SEKDEL/His6. To construct such vectors, the DNA piece encoding D3EIII (amino acid 300–420 of D3EIII, strain#16,881), along with its 3' prime sequence, was increased via PCR. This amplification utilized pTrcHisA-D3EIII [20] as a template, with primer 1 incorporating KpnI at its 5' end and primer 2 encompassing SEKDEL [21], His6 tag with SacI at its 3 prime end. Furthermore, other distinct 5' prime sequences, each covering nineteen amino acids of Pisum vicilin SP [22], along and without an additional 68 base pairs corresponding to the 5 prime UTR of TMV specific RNA, and the δ enhance component [23], were further amplified from artificial oligonucleotides incorporating KpnI and SacI at their 5 and 3 prime ends, respectively.

Subsequently, the PCR yields of each piece were further cloned into an Easy vector (pGEM-T ,Promega, USA) and subjected to sequence verification. The fragment encoding D3EIII/SEKDEL/His6 was then broken down with KpnI and SacI and embedded with SP or δ /SP, which had been similarly digested and sub-cloned into the TocJ vector. This TMV plant expression vector [12, 24] at the SacI site contained a T7 promoter, resulting in the generation of pSP/D3EIII/SEKDEL/His6 and p/SP/D3EIII/SEKDEL/His6 expression cassettes (Fig. 1).

2.2. Western blot analysis

Western blot analysis of samples vaccinated with either Normal or recombinant TMV-virus was conducted. Protein homogenates obtained from these plants were subjected to separation via 15% SDS-PAGE, following which the disjointed proteins were visualized using Coomassie Blue staining or transferred onto a PVDF membrane through electroblotting. Subsequently, the membranes were explored with either monoclonal antibodies against the His tag or a polyclonal antibody beside D3EIII. The anti-D3EIII polyclonal antibody was generated in rats through inoculation with refined D3EIII protein produced in Escherichia coli [24]. Following this, the membranes were additionally nurtured with HRP-conjugated antibodies; specifically sheep anti-mouse IgG or goat anti-rat IgG. Explicit binding interactions were visualized using an ECL detection system (Amersham Biosciences).

2.3. Refinement of D3EIII protein

The D3EIII protein purification process commenced with the harvesting of plant samples at 8 days post-inoculation (dpi), followed by extraction with binding buffer at a ratio of 2 ml per gram of extract from leaf. D3EIII testers were then subjected to purification through IMAC utilizing HiTrap Chelating-HP columns as per the instructions provided. The extracts were then applied to an IMAC column having binding buffer, followed by washing with a buffer containing 10 mM imidazole. Elution of D3EIII occurred in a sequential manner using elution buffer containing 30, 50, 110, 250, and 500mM imidazole concentrations.

The resultant elution fractions containing purified D3EIII protein were concerted using a concentrator and subsequently kept at -25°C. To verify the purity and integrity of the purified D3EIII protein, electroblotting on PVDF membranes was performed, followed by staining with Coomassie Blue. Subsequently, the purified protein bands were excised and subjected to sequencing.

2.4. Detection of D3EIII in inoculated tobacco by ELISA

The quantification of D3EIII protein levels was conducted through ELISA. Soluble samples were extracted and a Ninety-six well plate was coated with purified

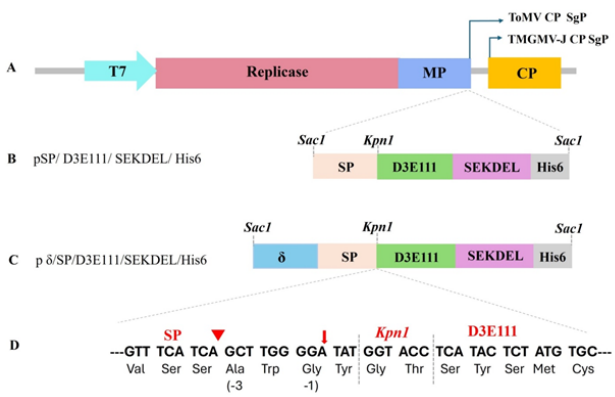

Fig. 1. TocJ genome schematic with insertion of D3EIII from dengue virus. D3EIII inserted at SacI site after CP gene. Nucleotide and protein sequences are shown.

bacterial D3EIII protein along with extracts from uninoculated plants. In brief, 200 ng of purified recombinant D3EIII was loaded on 96-well plates in each well and left overnight at 4°C. Subsequently, 5% skimmed milk in PBS to block the samples for 1 hour which were further incubated with serially diluted serum samples for 1 hour at 37°C. The Bound IgG protein was detected using HRP-conjugated goat anti-mouse IgG. Following the addition of TMB, a 450 nm wavelength was used for absorbance measurement. A value exceeding the mean +2 standard deviations of the negative control was taken as positive.

2.5. Immunization of mice

Female Balb/c mice, aged 4-6 weeks were divided into groups. Intramuscular injections were administered to one thigh of each mouse, with 10 micrograms of the refined D3EIII protein in the initial four immunizations on days 0, 21, 50, and 64. Subsequent immunizations on days 80, 87, 101, 115, and 128 involved administering 20 micrograms of the refined D3EIII protein in buffer. Blood were collected from mice on days 0, 20, 40, 60, 80, 100, 120, and 140. Additionally, blood was collected from mice on day 140. Serum obtained from each mouse was exposed to various immune assays.

2.6. Detection of D3EIII mouse serum IgG by ELISA

Serum IgG antibodies specific to virus-like particles were assessed through titration based on their binding affinity with recombinant EIII protein.. The IgG titers were determined by means of ELISA. Briefly, purified recombinant D3EIII was loaded on 96-well plates in each well and left overnight at 4°C. Subsequently, 5% skimmed milk in PBS to block the samples for 1 hour which were further incubated with serially diluted serum samples for 1 hour at 37°C. The Bound IgG protein was detected using HRP-conjugated goat anti-mouse IgG. Following the addition of TMB, a 450 nm wavelength was used for absorbance measurement. A value exceeding the mean +2 standard deviations of the negative control was taken as positive.

2.7. Neutralization Assay

To determine the quantity of neutralizing antibodies against dengue virus, the plaque reduction neutralization test was conducted. The PRNT protocol followed a method [29], with the utilization of dengue 3 virus, and monkey cells from kidney. The counteracting antibody titer was established as the uppermost serum dilution that compact the number of input plaques by 90-95%.

3. Results

3.1. Production and molecular analysis of purified D3EIII protein

For the production of a large quantity of purified D3EIII protein intended for the immunization of mice, the RNA products from the TMV construct δ/SP/DIII/KDEL/H, known for its extraordinary D3EIII synthesis levels, were injected into *N. tabacum* plants. To maximize D3EIII manufacture, the increase of the recombinant protein was first watched over time after inoculation. The ELISA analysis utilizing rat-specific D3EIII antibody revealed a gradual upsurge in D3EIII protein accumulation from 7 to 10 days post-inoculation (Fig. 2).

However, unembellished mosaic symptoms began to appear, causing the infested leaves to shrivel after 8 days post-infection, thereby complicating the collecting process. Consequently, it was determined that eight days post-infection was the optimum time for collecting infested plant material confirmation of normal and recombinant TMV replication in injected plants was achieved by observing the phenotype of a band conforming to around 18 kDa TMV external coat protein in the vaccinated tissues (Figure. 3A). For significant manufacture purposes, leaves homogenates displaying complete infection following the early inoculation with RNA was utilized to vaccinate new plants. The homogenates obtained from the primarily infected plants were infectious, resulting in the construction of D3EIII in leaves exhibiting general infection after the second infection. Notably, the D3EIII protein production level in inoculated leaves was found higher than that observed in leaves displaying systemic infection (Fig. 3). As anticipated, no sign was noticed in non-inoculated leaves. Our findings indicate the stability of the virus and its retention of contagion upon passageway to the second level of infection. Moreover, TocJ-based vectors exhibit the ability to express a foreign protein systemically but also

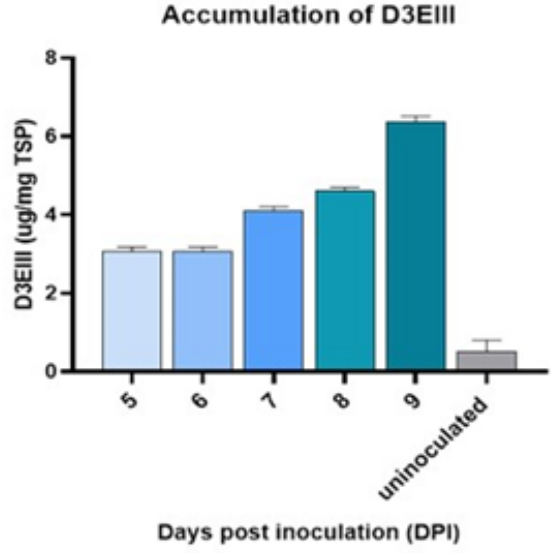

Fig. 2. Recombinant D2EIII protein accumulation in *N. tabacum* plants infected with δ/SP/D2EIII/SEKDEL/His6 was monitored over 5–9 days post-infection (dpi). Samples were analyzed via indirect ELISA to quantify protein levels in crude protein extracts, with uninoculated plants serving as controls.

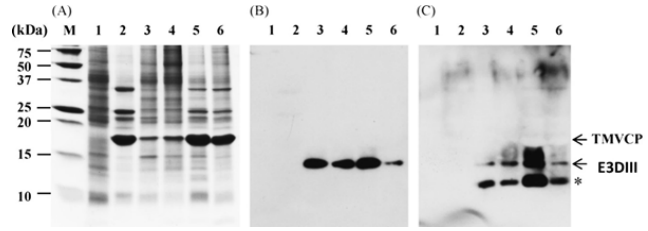

Fig. 3. Analysis of recombinant D3EIII accumulation in *N. tabacum* plants infected with δ/SP/D3EIII/SEKDEL/His6 was conducted using Coomassie Brilliant Blue staining (A) or Western blot analysis with anti-His tag Mab (B) and rat anti-D3EIII polyclonal antibody (C). Plant extracts (10μg) were isolated from various leaves, including uninoculated, TocJ, wild-type TMV infected, primary infected, and secondary infected leaves. Lane M represents the molecular weight marker (Precision Plus Protein Standards, BioRad, USA). An asterisk denotes potential degradation of plant-produced D3EIII protein.

the capability to maintain the external sequences intact through passage from one plant to another. The recombinant D3EIII obtained from secondarily infested leaves was purified under normal conditions using IMAC.

Furthermore, purified D3EIII protein, with a molecular weight of 13.8 kDa, was obtained through elution with a buffer containing 100mM imidazole (Figure 4) and demonstrated reactivity to both anti-His tag and anti-D3EIII antibodies (Figure 4).

3.2. Stimulation of anti-D3EIII antibody production in mice following immunization with refined D3EIII

To assess the immunogenicity of the purified D3EIII protein, protein was injected into different mice intramuscularly together with TiterMax. The induction of antibodies targeting dengue virus was evaluated using a dengue IgG ELISA system, wherein the well plate was coated with dengue virus antigens (serotypes 1–4). Sera collected at various time points during the immunization regimen were tested for the presence of anti-dengue virus antibodies. Our results indicated that mice immunized with the D3EIII protein generated anti-dengue-specific virus antibodies, with notably greater levels observed in mice numbered three and four (Fig. 5).

In mice three and four, antibody levels were initially little during the early phase of inoculation with 12 micrograms per dose. However, antibody levels increased rapidly after days 70-80 when we increased the antigen dosage to 20 micrograms. Our findings suggest that the plant-specific D3EIII is capable of eliciting dengue-specific antibodies in mice. These serums were also analyzed for the presence of anti-D3EIII antibodies via western blot. The results indicated that mouse serum exhibited reactivity with both purified D3EIII protein and extracts, showing solid reactivity at a dilution of one into thousands (Fig. 6), while displaying no reactivity towards non-inoculated leaves or wild-type (Fig. 6).

4. Discussion

The TMV-based vector system offers several advantages for the production of DENV-3 EDIII in plants, including high-level expression, rapid production timelines, scalability, and proper protein folding and assembly. Moreover, plants serve as ideal bioreactors for the production of vaccines, as they can be easily scaled up to meet the demand for large quantities of protein. However, challenges such as low stability of recombinant proteins, potential toxicity of viral vectors, and regulatory concerns need to be addressed for the commercialization of plant-produced vaccines.

In this report, we detail our successful use of a *N. tabacum* TMV-based viral vector as a reactor for producing the D3EIII. We evaluated the D3EIII protein's ability to stimulate the synthesis of anti-dengue virus antibodies and neutralizing antibodies in contradiction of dengue virus 3

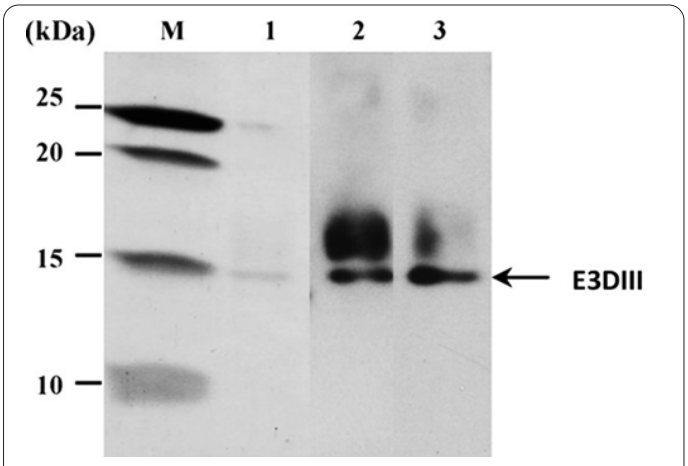

Fig. 4. SDS-PAGE and Western blot analysis of purified D3EIII protein. Extracts from inoculated leaves were purified using IMAC (HiTrap chelating column, Amersham Biopharmacia, UK). The purified D2EIII protein (0.25ug) from the fraction eluted with 100mM imidazole was detected by Coomassie Brilliant Blue staining (lane 1), Western blotting with anti-His tag Mab (lane 2), and rat anti-D2EIII polyclonal antibody (lane 3). Lane M represents the protein molecular weight marker (Precision Plus Protein Standards, BioRad).

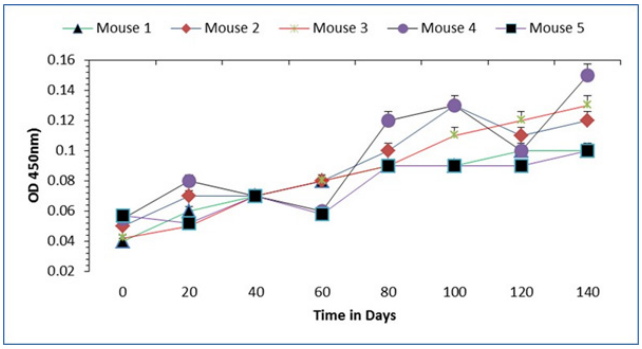

Fig. 5. Mice were immunized intramuscularly with purified D3EIII protein from N. benthamiana plants. Mouse sera collected during immunization were analyzed using ELISA to determine specific IgG antibodies against dengue virus types 1–4, using a dengue IgG indirect ELISA kit (PanBio, Australia). Each line on the graph represents the response of an individual mouse (Mouse Nos. 1–5).

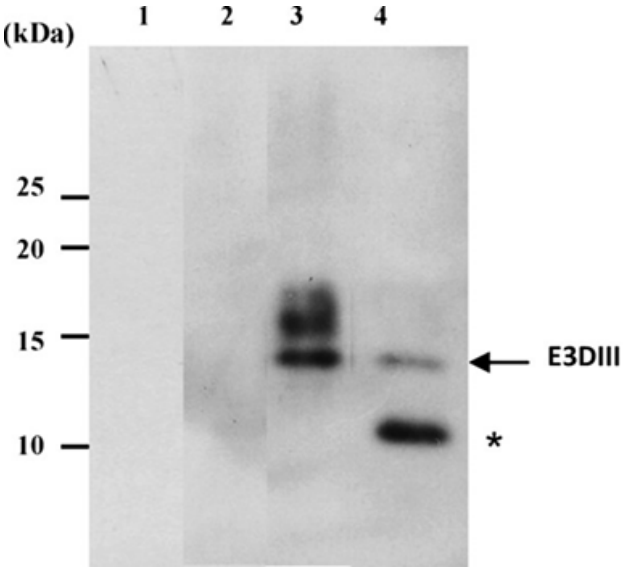

Fig. 6. Western blot analysis of infected plant samples was performed using mouse anti-D3EIII antibody. Protein samples underwent 15% SDS-PAGE and were probed with serum from mouse No. 3 (dilution 1:1000) and goat anti-mouse IgG-HRP conjugate (dilution 1:1000). Lane 1: uninoculated leaves (negative control, 10ug); lane 2: wild-type TMV, TocJ inoculated leaves (control vector, 10g); lane 3: purified plant-produced D3EIII protein (0.25g); lane 4: plant extract inoculated with δ/SP/DIII/KDEL/H (10g). The protein molecular weight marker (Precision Plus Protein Standards, BioRad) is displayed in the left panel.

when directed intramuscularly to mice. Results from the vaccination experiment demonstrated that the plant-based D3EIII protein effectively prompted the generation of anti-dengue virus antibodies and anti-D3EIII antibodies in mice. Notably, there was a positive correlation observed between the level of anti-dengue virus antibodies and the neutralizing antibody activity in the sera of immunized mice, particularly evident in mouse numbers 3 and 4.

However, the induction level of antibodies specific to the dengue virus antigen in the immunized mouse sera was relatively low (Figure 5). This could be attributed to the small size of the antigenic piece, resulting in inadequate stimulation of robust antibody construction, despite the use of an adjuvant in the experiment. Therefore, to ensure the production of neutralizing antibodies in immunized mice, repeated immunizations with the plant-based D3EIII protein along with TiterMax Gold adjuvant were administered over an extended period.

CTB has been demonstrated to be an actual transporter for vaccines and is extensively used as an adjuvant for oral vaccines [25]. Therefore, vaccine development efforts should prioritize the induction of high levels of antibody neutralization. Although the antibody neutralization titers persuaded by the plant-based D3EIII protein were not exceptionally high, they showed promise. Strategies such as adjusting antigen dosage, altering the route of immunization, or employing different adjuvants could potentially enhance neutralizing antibody titers. TMV-based vectors were initially developed and have since been refined to improve stability and increase foreign protein expression levels [26]. Further enhancements involved strategies to expand movement and expand host-ranges through DNA scuffling of the specific protein [27, 30, 31]. The benefits of using TMV as a vector system include extraordinary yields of external proteins, rapid protein expression, and low toxicity. Additionally, *N. tabacum* plants should be cultivated in fully isolated greenhouse facilities to prevent human interaction or access by birds and insects, with treated wastewater being the only waste released from the facility.

The production of purified D3EIII protein was 0.30% of TSP. In this study, the expression level of D3EIII in tobacco leaves surpassed that of the highly varied region 1, a probable neutralizing epitope of the hepatitis virus C, expressed in tobacco leaf using a similar expression system, as reported previously [8, 32]. Thus, it appears that the elevated expression level of D3EIII was attained through a grouping of factors, targeting the protein to the endoplasmic reticulum via the signal peptide and 5 prime untranslated regions. Despite partial degradation of the D3EIII protein during preparation from infected plants, the proteins remained immunoreactivity with anti-D3EIII antibodies (Figures 3, 6). During refinement by halted metal IMAC, merely whole D3EIII was isolated, with a yield of 0.30% of TSP.

5. Conclusion

In conclusion, we have demonstrated that the TMV-based vector is highly effective for producing recombinant D3EIII protein in Nicotiana tabacum plants. The recombinant protein retains its immunogenicity and successfully induces neutralizing antibodies in immunized animals. This study marks the first successful use of the TMV-based expression system to produce recombinant D3EIII proteins that effectively stimulate immune responses in developed models. However, further enhancements are necessary to improve the immunological properties of the recombinant proteins.

Conflicts of interest

There are no conflicts to declare.

Ethical approval

Approval for this study was granted by the research ethics committee at the Health Science Research Center, King Abdullah bin Abdulaziz University Hospital (IRB Log Number : 23-0020), Riyadh, Saudi Arabia. The research adhered to the principles outlined in the " Guide for the Care and Use of Laboratory Animals," as issued by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

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