Rational Design of Dengue Vaccines: Integrating Mutagenesis, Structure Prediction, and Docking Simulations in *Nicotiana tabacum*

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A. BACKGROUND

Dengue fever, also known as break-bone fever, is a viral illness caused by the dengue virus (DENV) and is primarily transmitted to humans through the bites of infected female mosquitoes, particularly *Aedes aegypti* and its secondary vector, *Aedes albopictus*. This disease is especially prevalent in tropical and subtropical regions, including Indonesia, where dengue has been endemic for years (Arguni *et al.,* 2022). Belonging to the Flaviviridae family, which also includes viruses like Chikungunya, Yellow fever, and Zika virus, dengue viruses are categorized into four distinct serotypes: DENV-1, DENV-2, DENV-3, and DENV-4. Each serotype has unique antigenic and phylogenetic properties. While infection with one serotype provides immunity against that specific strain, it does not confer protection against the others, thereby increasing the global burden of the disease (Bhagavan, 2020). The transmission cycle begins when a female mosquito feeds on an individual infected with DENV, allowing the virus to replicate in the mosquito's midgut before spreading to its other tissues, including the salivary glands. After an Extrinsic Incubation Period (EIP) of 8–12 days under optimal temperatures (25–28°C), the mosquito becomes infectious and can transmit the virus throughout its lifespan. Environmental factors such as temperature variations, virus genotype, and initial viral load can influence the duration of the EIP. Urban environments and artificial water containers, such as plastic bins and old tires, serve as ideal breeding grounds for Aedes mosquitoes, exacerbating the spread of dengue. Recent global challenges have further heightened the risk of dengue outbreaks, including the shifting distribution of vector mosquitoes due to climate change, the 2023 El Niño phenomenon that increased rainfall, humidity, and temperatures, and weakened healthcare systems strained by the COVID-19 pandemic. Additionally, political and financial instability in regions experiencing humanitarian crises has further complicated efforts to mitigate the impact of dengue (WHO, 2024)

Although commonly known to cause classical dengue fever, the dengue infection also results in a whole spectrum of illness ranging from asymptomatic infection with no clinical presentation to severe forms of infection. Several factors contributing to the severity of dengue infection include secondary and concurrent infection, and also the serotype of dengue virus. According to 1997 WHO classification, severe dengue infections include dengue haemorrhagic fever and dengue shock syndrome. However, in 2009 the WHO redefined both dengue haemorrhagic fever and dengue shock syndrome simply as severe dengue infection, which are characterized by severe bleeding, plasma leakage, and severe organ impairment. Severe dengue infection leads to higher mortality rate (Soo *et al*., 2016).

Based on data from the Sleman District Health Office (interview questions can be seen in **Table S1** in supplementary material), dengue fever cases in 2023 was 146 cases. Meanwhile, in 2024, dengue fever cases increased to 675 cases, three times more than the previous year. Based on the data, dengue fever cases in Yogyakarta also reached 2000 cases. High dengue fever cases are caused by various interrelated factors, for instance environmental conditions, virus transmission, the presence of vectors, and the presence of hosts. Environmental conditions such as high humidity and El Nino increase the mosquitoes' ability to mate. On the other hand, high human mobility can also increase the spread of the dengue virus that causes dengue fever. Dengue virus (DENV) contains four distinct serotypes, each with their own antigenic features. According to earlier studies on the prevalence of these serotypes, DENV-2 and DENV-3 are the most common in Yogyakarta.

DENV-3 has the highest prevalence nationwide (Rahayu *et al,* 2019; Arguni *et al*., 2022; Masyeni *et al*., 2023; Utama *et al*., 2019; Sasmono *et al*., 2020).

There are several programs from the government to overcome dengue, but not enough to overcome it. One solution is vaccination, but the dengue vaccine production process is still quite expensive. In addition, the vaccine that has been approved by the Indonesian government is the Live-Attenuated recombinant vaccine, both of which are produced in vero cells (Angelin *et al*., 2023; Thoomas and Yoon, 2019). The ideal vaccine manufacturing chassis is still being sought after. Chung *et al*. (2021) suggested using plants as bioreactors to produce vaccines. Because plants reduce the possibility of contamination and promote appropriate protein folding. Molecular farming provides a safe, scalable, and affordable alternative for the manufacture of vaccines. It is perfect for global health applications since it also facilitates quick vaccine development, is environmentally friendly, and lessens reliance on cold chains. We created a subunit vaccine expression circuit using a *Nicotiana tabacum as* chassis in order to solve this issue. Furthermore, in order to address the issue of protein shape instability, we looked for mutagenesis sites in order to optimize the protein shape and assess the optimal vaccine design. By using plants as a bioreactor for the creation of recombinant proteins, this study seeks to address the difficulties in vaccine production. In order to ensure improved efficacy and stability of the vaccine candidate, this study also aims to optimize protein structural stability using site-directed mutagenesis evaluation.

B. MATERIALS AND METHODS

a. Computational and Experimental Workflow in Protein Engineering: From Site-Directed Mutagenesis to Free Energy Binding Analysis

We performed a literature review to find possible mutagenesis sites utilising a variety of databases prior to starting the computational analysis. We gather information on mutagenesis sites and the accession codes that correspond to them. Our literature review findings are shown in **Table 1.**

Table 1. Literature Result of Mutagenesis Site

Serotype	Mutagenesis Site	PDB Accession Code	Sources
DENV2	- K305A and P384A (type-specific antigenic sites) - K310A (DENV complex-reactive sites)	1OAN	Pitcher *et al*., 2015
DENV3	- L306 - K308 - G381 - I387 - W389	1UZG	Matsui *et al*., 2009

Based on knowledge gathered from peptide scanning, bioinformatics analysis, and crystal structure evaluation, site-directed mutagenesis was carried out to look at the functional roles of important amino acid residues in protein vaccines stability aspects. The RCSB Protein Data Bank provided the protein sequences used in this investigation, namely 1OAN (Dengue virus serotype 2) and 1UZG (Dengue virus serotype 3). Because of their significant frequency in Indonesia, especially in the Yogyakarta region, DENV2 and DENV3 were selected. Zero-shot variant prediction with protein language models, specifically ESM2, was utilized to predict the relative fitness of protein variants and to derive mutagenesis insights. This method quantifies the likelihood of mutations by comparing the probability

scores assigned to wild-type and mutant amino acids at specific sequence positions. By integrating evolutionary constraints on protein function, the approach provides robust predictions of the potential functional impacts of mutations, offering valuable insights into their influence on protein activity, stability, and overall fitness.

Following mutagenesis, VaxiJen v2.0 and AllerTop were used to evaluate for antigenicity and allergenicity after mutagenesis. While allergenicity tests made sure that there were no allergenic sequences, which improved the constructs safety and appropriateness for future development, antigenicity tests assessed the constructs capacity to generate a robust immune response. Sequences having antigenicity meet the inclusion criteria, whereas sequences with allergenicity meet the exclusion requirements.

ColabFold v1.5.5, an implementation of AlphaFold2 with MMseqs2, was used to predict the three-dimensional (3D) structures of the vaccine constructs. A number of parameters were set to optimise the prediction: template_mode to pdb100, msa_mode to mmseqs2_unirev_env, pair_mode to unpaired_unpaired, model_type to alphafold2_multimer_v3, num_recycles to 24, relax_max_iterations to 2000, and pairing_strategy to complete. Full parameters and step by step can be found in the **S5 Method** in supplementary material. Then, protein-protein docking simulations were carried out using ClusPro to examine interactions between vaccine candidates and target receptor proteins, and the docking results made it easier to identify the most stable and functionally relevant vaccine-receptor complexes for additional molecular dynamics analysis.

Molecular dynamics simulations were used to evaluate the vaccine-receptor complexes stability and interaction dynamics. CHARMM-GUI was used to prepare the sample, and then a suitable molecular dynamics software program was used for simulations.

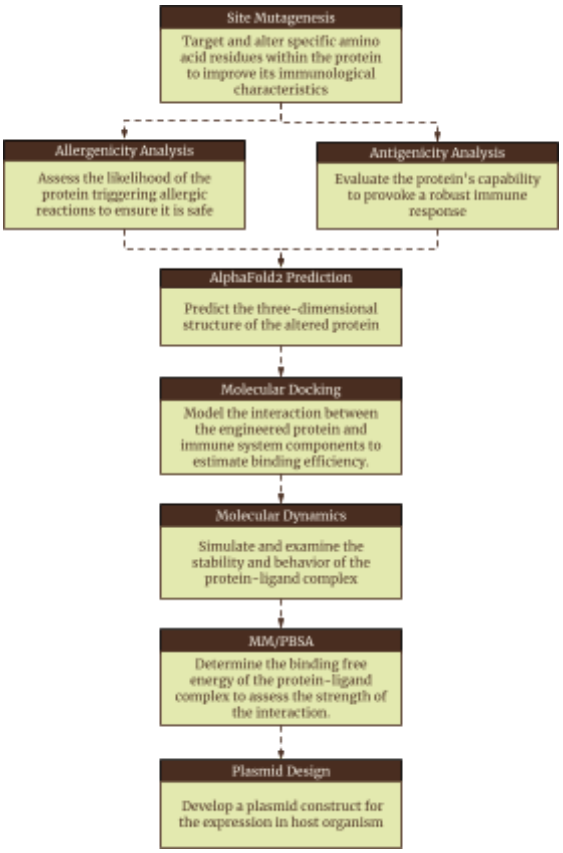


Figure 1. Workflow diagram of Computational and Experimental in Protein Engineering

We use gmx_MMPBSA v1.5.x to perform MMPBSA (Molecular Mechanics Poisson-Boltzmann Surface Area) calculations for estimating the binding free energies of

non-covalently bound complexes. MMPBSA is a widely used computational method that combines molecular mechanics energy terms with solvation models (Poisson-Boltzmann or Generalized Born) and surface area calculations to approximate the binding free energies in molecular complexes (citation). This method utilizes GROMACS files for simulating the interactions between molecules, enabling detailed and efficient analysis of the energetics of ligand-receptor binding. Trajectory files from molecular dynamics simulations served as the basis for these computations. This method offered important insights into the possible effectiveness of several vaccination candidates by statistically assessing the binding affinity between vaccine constructs and their target receptors.

The MM/PB(GB)SA approach uses molecular dynamics (MD) simulations to estimate the binding free energy (Δ*Gbind*) of complexes that are not covalently bound. This is how the free energy is computed: **(1)** $\Delta G_{bind} = \langle G_{COM} \rangle - \langle G_{REC} \rangle - \langle G_{LIG} \rangle$, where each term is expressed as: **(2)** $\langle G_x \rangle = \langle E_{MM} \rangle + \langle G_{sol} \rangle - \langle TS \rangle$. Alternatively, **(3)** $\Delta G_{bind} = \Delta H - T\Delta S$, where entropy loss upon ligand binding is taken into account by $-T\Delta S$ and ΔH stand for enthalpy. The effective free energy is calculated for ligand comparison when entropy is disregarded. The enthalpy term is: **(4)** $\Delta H = \Delta E_{MM} + \Delta G_{sol}$, where the free energy contributions from the gas phase are: **(5)** $\Delta E_{MM} = \Delta E_{bonded} + \Delta E_{nonbonded} = (\Delta E_{bond} + \Delta E_{angle} + \Delta E_{dihedral}) + (\Delta E_{ele} + \Delta E_{vdW})$. For solvation energy ($\Delta G_{sol}$) is: **(6)** $\Delta G_{sol} = \Delta G_{pol} + \Delta G_{non-pol} = \Delta G_{PB/GB} + \Delta G_{non-pol}$, where **(7)** $\Delta G_{non-polar} = NP_{TENSION} * \Delta SASA + NP_{OFFSET}$, or **(8)** $\Delta G_{non-pol} = \Delta G_{disp} + \Delta G_{cavity} = \Delta G_{disp} + (CAVITY_{TENSION} * \Delta SASA + CAVITY_{OFFSET})$ (Miller *et al*., 2012).

b. Optimization, Cloning, and Plasmid Construction of a Modified Hyperexpression System for Plant-Based Vaccine Production

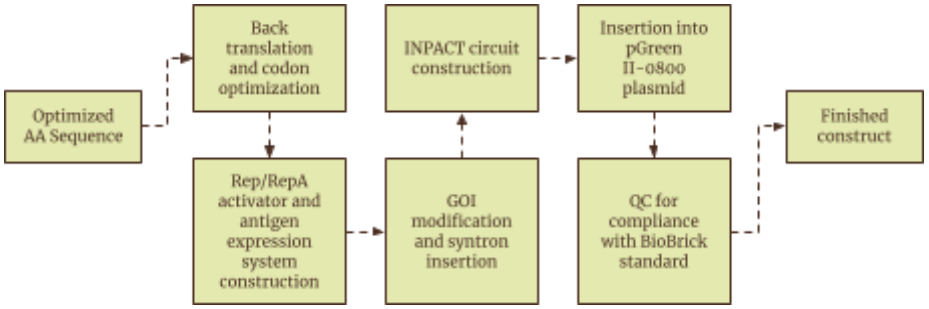


Figure 2. Design workflow, modified protocol based on Dugdale *et al*. (2014).

Vaccine sequences were first back translated and codon optimized then cloned into a modified expression system based on the In-Plant activation system developed by Dugdale, et al. (2013) from Tobacco Yellow Dwarf Virus (TYDV). The system utilizes the rolling circle replication mechanism found in TYDV to increase recombinant protein yield via hyperexpression, and has been successfully implemented for expression of various proteins including GUS, human vitronectin, barnase, and various other proteins in *Nicotiana tabaccum.* Our system substitutes the ethanol inducible AlcR/PalcA system upstream of the Rep/RepA gene with a salt-inducible synthetic promoter. Additionally, both the Rep/RepA activator gene and the INPACT cassette containing the bivalent vaccine are inserted into the same binary Ti plasmid backbone (pGreenII-0800) to ensure co-infiltration of both genes and streamline the transformation process.

C. RESULT

a. Vaccine Construct Result

Five best mutant generation results presented in **Table 2**, were combined to create the vaccine construct. Through allergenicity and antigenicity evaluation, we obtained four

vaccine constructs for protein-receptor interaction analysis. The results of four mutant vaccine constructs and the native vaccine construct are compared, as shown in **Table 4.**

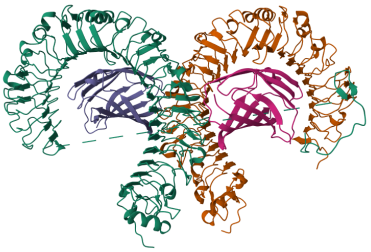
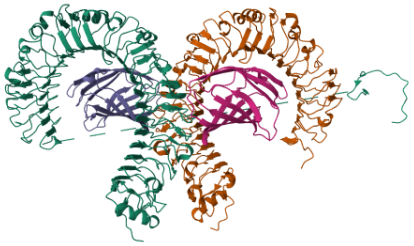
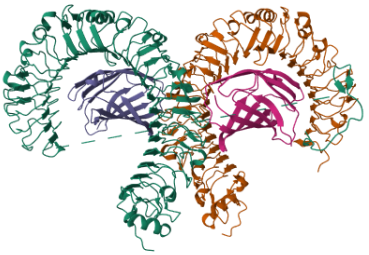
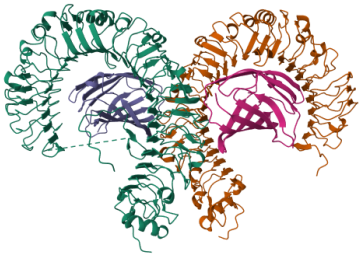
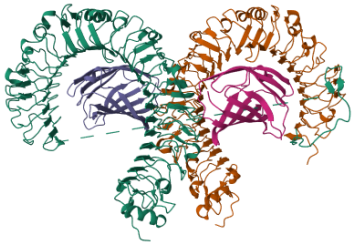
Table 2. Amino Acid Sequences of Native and Mutant Protein E Domain III

DENV2 Protein E Domain III	**DENV3 Protein E Domain III**
Native (1OAN) GMSYSMCTGKFKVVKEIAETQHGTIVIR VQYEGDGSPCKIPFEIMDLEKRHVLGRLI TVNPIVTEKDSPVNIEAEPPFGDSYIIIGVE PGQLKLNWFKK	Native (1UZG) SYAMCLNTFVLKKEVSETQHGTILIKVE YKGEDAPCKIPFSTEDGQGKAHNGRLIT ANPVVTKKEEPVNIEAEPPFGESNIVIGIG DKALKINVYRK
D2M1 GMSYSM<u>E</u>TGKFKVVKEIAETQHGTIVIR VQYEGDGSPCKIPFEIMDLEKRHVLGRLI TVNPIVTEKDSPVNIEAEPPFGDSYIIIGVE PGQLKLNWFKK	D3M1 SYAMCLNTFVLKKEVSETQHGTILIKVE YKGEDAPCKI<u>T</u>FSTEDGQGKAHNGRLIT ANPVVTKKEEPVNIEAEPPFGESNIVIGIG DKALKINWYRK
D2M2 GMSYSMCTGKFKVVKEIAETQHGTIVIR VQ<u>N</u>EGDGSPCKIPFEIMDLEKRHVLGRLI TVNPIVTEKDSPVNIEAEPPFGDSYIIIGVE PGQLKLNWFKK	D3M2 SYAM<u>D</u>LNTFVLKKEVSETQHGTILIKVE YKGEDAPCKIPFSTEDGQGKAHNGRLIT ANPVVTKKEEPVNIEAEPPFGESNIVIGIG DKALKINWYRK
D2M3 GMSYSMCTGKFKVVKEIAETQHGTIVIR VQYEGDGSP<u>V</u>KIPFEIMDLEKRHVLGRLI TVNPIVTEKDSPVNIEAEPPFGDSYIIIGVE PGQLKLNWFKK	D3M3 SYAMCLNTFVLKKEVSETQHGTILIKVE YKGEDAP<u>V</u>KIPFSTEDGQGKAHNGRLIT ANPVVTKKEEPVNIEAEPPFGESNIVIGIG DKALKINWYRK
D2M4 GMSYSMCTGKFKVVKEIAETQHGTIVIR VQYEGDGSPCKIPFEIMDLEKR<u>K</u>VLGRLI TVNPIVTEKDSPVNIEAEPPFGDSYIIIGVE PGQLKLNWFKK	D3M4 SYAMCLNTFVLKKEVSETQHGTILIKVE YKGEDAPCKIPFSTEDGQGKA<u>V</u>NGRLIT ANPVVTKKEEPVNIEAEPPFGESNIVIGIG DKALKINWYRK
D2M5 GMSYSMCTGKFKVVKEIAETQHGTIVIR VQYEGDGSPCKIPFEIMDLEKRHVLGRLI TVNPIVTEKDSPVNIEAEPPFGDSYIIIGVE PGQLKLN<u>L</u>FKK	D3M5 SYAMCLNTFVLKKEVSETQHGTILIKVE YKGEDAPCKIPFSTEDGQGKAHNGRLIT ANPVVTKKEEPVNIEAEPPFGESNIVIGIG DKALKIN<u>V</u>YRK

Table 3. Positions and Mutant Score of Top 5

DENV2			**DENV3**		
Position	**Mutant**	**Scores**	**Position**	**Mutant**	**Scores**
302	C302E	2.895199537	334	P334T	2.600211143
326	Y326N	2.68605566	300	C300D	2.385468245
333	C333V	2.19573164	331	C331V	2.383506775
346	H346K	2.183547497	345	H345V	1.995705843
391	W391L	1.946171284	389	W389V	1.966603994

Table 4. Assessment Result of the Final Construct's Allergenicity, Antigenicity, Interaction and Free Energy Binding Prediction

Vaccine Construct	Allergenicity & Antigenicity	Structure Interaction	Δ Total
D2Native_D3Native	Non-Allergen Antigen (0,5967)		-91.36
D2M1_D3M1	Non-Allergen Antigen (0,6263)		-118.89
D2M1_D3M3	Non-Allergen Antigen (0,6147)		-72.53
D2M2_D3M1	Non-Allergen Antigen (0,6200)		-128.01
D2M3_D3M3	Non-Allergen Antigen (0,6121)		-90.21

b. Plasmid Design Result

Plasmid map result from the design steps mentioned above is as depicted in **Figure 3.** Total length of the insert is 3829 bp and the total length of both insert and backbone is 6402 bp.

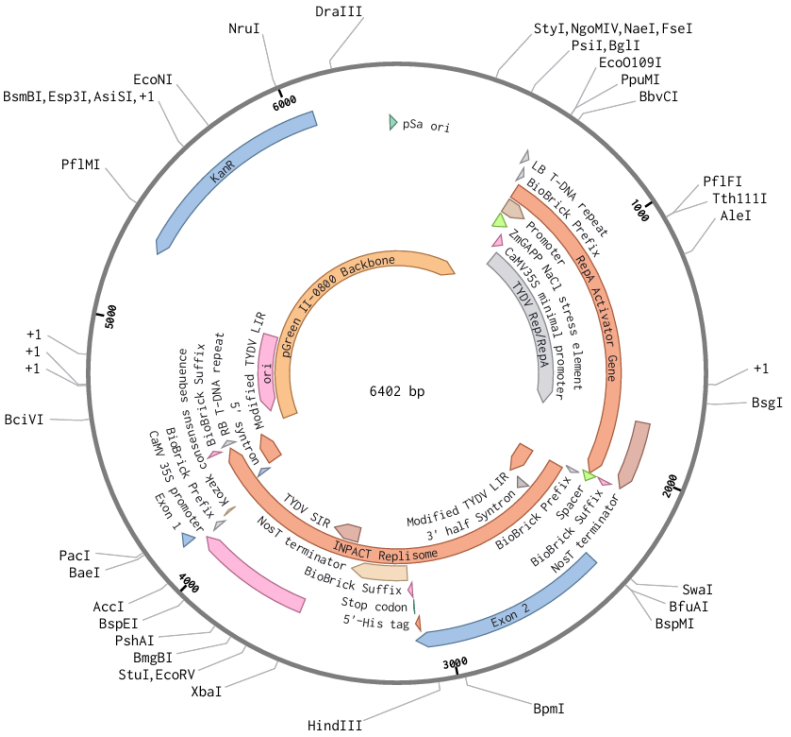


Figure 3. Plasmid map of the bivalent dengue vaccine expression system.

D. DISCUSSION

Site-directed mutagenesis is a widely utilized method for elucidating the roles of key amino acid sites identified through preliminary screenings such as peptide scanning, bioinformatics analysis, and crystal structure analysis. This technique entails the substitution of specific amino acid residues, followed by an examination of the effects on the mutant peptide or the biological function of the virus, ultimately facilitating the identification of these critical sites (Cui *et al*., 2020). To enhance our method, we decided to implement a more modern model called ESM-2.

This model, which is a Large Language Model (LLM) similar to ChatGPT, is specifically trained on amino acid sequences rather than text from the internet. ESM-2 is capable of capturing long-range dependencies and evolutionary relationships in protein sequences, making it particularly effective for tasks such as structure prediction and functional annotation. Studies have shown that ESM models outperform traditional methods in predicting highly active protein variants (citation). Using the zero-shot capability of ESM-2, we generated mutagenesis data and obtained scores that predict the relative fitness of mutants. These scores provide valuable insights into how specific mutations might affect protein function, enabling us to prioritize variants for further experimental validation.This is achieved by comparing the probability predicted by ESM-2 for the occurrence of the mutant amino acid to that of the wild-type amino acid at the same location. This method leverages the understanding that evolution is constrained by protein function (Meier et al., 2021); however, there is still uncertainty regarding its ability to generalize to non-native functions.

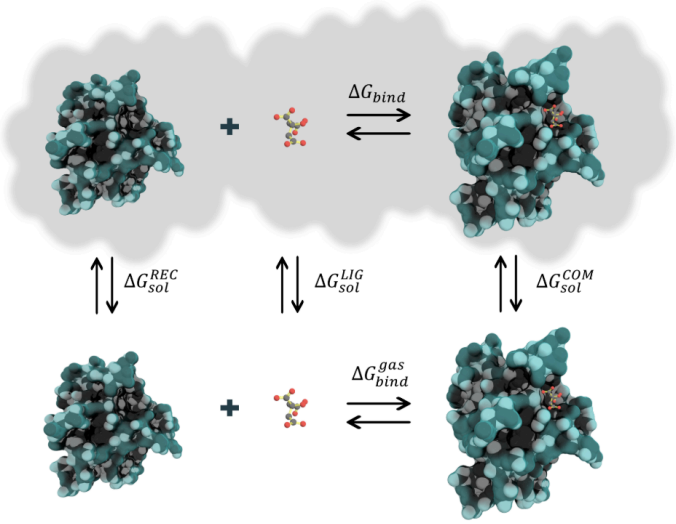


Figure 4. Thermodynamic cycle for binding free energy calculations (Miller *et al*., 2012)

Notable high-scoring mutations include C302E in DENV2 (2.895) and C300D in DENV3 (2.385), which indicate a predicted beneficial impact on protein fitness. For the top 5 score mutations can be found in **Table 3**. We use the top 5 in each serotype and make 25 probability of both combinations. These mutations can be further investigated to optimize peptide sequences for improved structural and functional properties (Cheng *et al*., 2024). The sequences of the mutation results and native sequences can be seen in **Table 2** and the location of the all mutant generation results can be seen in **Table S2** in the supplementary material.

Concurrently, vaccine constructs derived from these sequences undergo evaluation for antigenicity and allergenicity using VaxiJen v2.0 and AllerTop V.2.0. Antigenicity assessments predict the potential of vaccine constructs to elicit strong immune responses, promoting long-term immunological memory (Barazesh *et al*., 2024) while allergenicity evaluations ensure the absence of allergenic sequences, thereby enhancing safety (SobhZahedi and YektaKooshali, 2024). All sequences exhibit a lack of allergenic properties and demonstrate a promising antigenic score. For all results, please see **Table S4** in the supplementary material.

ColabFold v1.5.5: AlphaFold2 with MMseqs2 [(Kim et al., 2024)].(https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb) was used to model the vaccine's 3D structure. The results of the predicted vaccine-receptor interactions can be seen in **Table 4**, complete with visualization of the structure and Δ Total of the free binding energy. ClusPro was then used to predict interactions between receptor protein complexes and vaccination proteins. After receiving the PDB file, molecular dynamics simulations were run to create trajectory files for subsequent analysis with MMPBSA. The vaccine-receptor complex's stability and interaction dynamics can be further assessed using parameters such as **root-mean-square RMS**, **solvent-accessible surface area (SASA)**, and **radius of gyration (Rg)**.

Data of free energy binding capacity was obtained from Molecular Mechanics Poisson-Boltzmann Surface Area (MM/PBSA). Native was found to have an average of Δ total at -91.36 kcal/mol, while the average of Δ total of mutants varies from 20% higher (-72.52 kcal/mol for DENV-2 mutant 1 + DENV-3 mutant 3) to 40% lower (-128.01 kcal/mol from DENV-2 mutant 2 + DENV-3 mutant 1). The lower the free energy binding capacity is the easier for ligand and receptor to form protein-protein complex. Thus, from MMPBSA there are 2 better candidates than native, which are DENV-2 mutant 2 + DENV-3 mutant 1

with -128.01 kcal/mol for an average of Δ total and DENV-2 mutant 1 + DENV-3 mutant 1 with -118.89 kcal/mol for an average of Δ total. The full data can be found in **Table 4**.

Finally, we constructed plasmid circuits in pGreen for the *Nicotiana tabacum* chassis system. In this study, we modified the existing TYDV In-Plant Activation (INPACT) system to include a salt-inducible synthetic promoter upstream of the Rep/RepA gene, utilizing the ZmGAPP promoter as depicted in **Figure 5**. This modification aims to improve co-infiltration and transformation efficiency, ensuring robustness and tightness. To improve translation efficiency in Nicotiana tabacum, codon optimisation was performed using Benchling with the default parameters. The ZmGAPP-based Rep/RepA activation system was chosen because of its well-defined 71 bp salt-sensitive regulatory sequence (Hou et al., 2016) and compatibility with the 35S minimum promoter (Amack & Antunes, 2020). The use of the powerful CaMV 35S promoter to induce antigen production, together with the presence of a Kozak motif within the 5'-UTR, guarantees that translation is initiated efficiently. Furthermore, both the Rep/RepA and antigen genes used the NosT terminator, which promotes transcript stability and effective termination. The vaccine ORF was divided into two exons separated by a synthetic intron (syntron) carrying the TYDV LIR (Dugdale et al., 2014) to leverage intron-mediated enhancement (IME), a mechanism known to significantly boost gene expression in plants. To further enhance expression, we introduced BioBrick prefix and suffix sequences that comply with RFC standards, allowing for standardized assembly and modularity in future modifications. We shortened the transformation and expression process by cloning the Rep/RepA and vaccination genes into pGreenII-0800. Gibson primers were designed for smooth assembly using NEB Gibson Assembly, which permitted exact build integration. The inclusion of structural components at consensus splice sites was a critical method for increasing IME-driven expression and ensuring adequate antigen production. Overall, our improvements to the TYDV INPACT system provide a refined approach to plant-based vaccine development, balancing rigorous regulatory control with increased expression levels. Future research should focus on in vivo validation, expression kinetics, and immunogenicity assessments to confirm the efficacy of this technique in large-scale vaccine production.

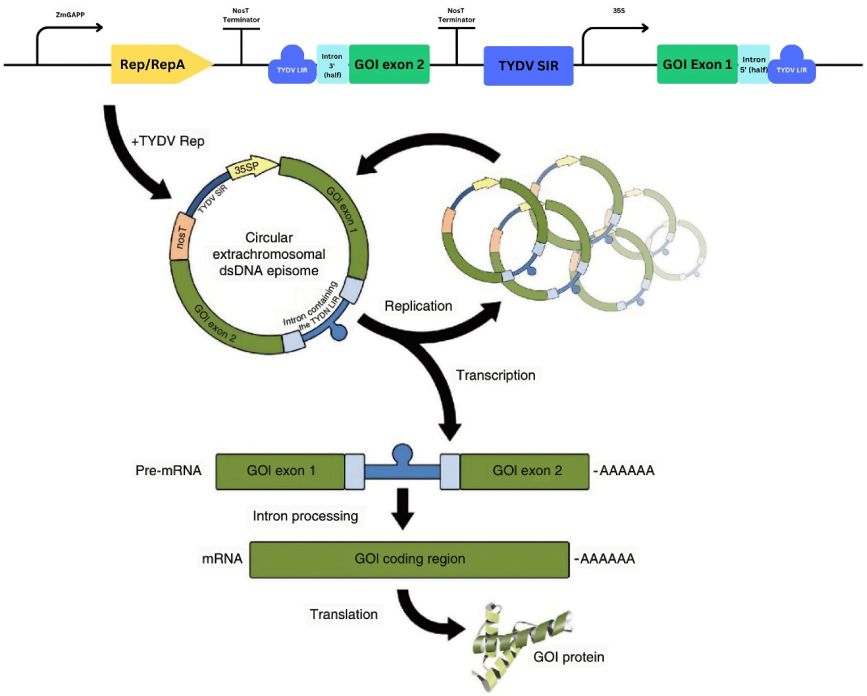


Figure 5. Schematic representation of a modified hyperexpression platform based on the INPACT system developed by Dugdale, *et al.* (2013).

E. CONCLUSION AND FUTURE WORK

Our experiments show that using site-directed mutagenesis to alleviate the instability of recombinant proteins produces considerable results. The optimal DENV-2 mutant 2 + DENV-3 mutant 1 with a binding energy of -128.01 kcal/mol was identified through a comprehensive workflow that included site-directed mutagenesis, antigen inclusion and allergen exclusion criteria, structural prediction using ColabFold, complex protein PDB acquisition via ClusPro, molecular dynamics file preparation using Charm_GUI, molecular dynamics simulations, and binding energy calculations with MMPBSA. This mutant was shown to be antigenic, non-allergenic, and had a consistent structural prediction. In addition, a circuit plasmid was created employing the pGreenII-0800 backbone as a production vector for expression in Nicotiana tabacum. This circuit includes essential elements such as the salt-inducible promoter ZmGAPP, the NosT terminator, the gene of interest (GOI) coding sequence, TYDV LIR, TYDV SIR, a syntron containing TYDV LIR (RNA region), and the CaMV 35S constitutive promoter. The effective creation of this plasmid illustrates its potential use in plant-based vaccine manufacturing. This study effectively modified the TYDV INPACT system and used site-directed mutagenesis to improve antigen stability in order to create an optimised expression system for the manufacturing of plant-based vaccines. Effective gene expression in *Nicotiana tabacum* was ensured by codon optimisation and strategic sequence alterations, while transformation efficiency was increased by combining the Rep/RepA activator gene and the INPACT cassette into a single plasmid backbone. The stability, antigenicity, and non-allergenic qualities of the developed vaccine candidates were validated by structural and molecular dynamics investigations. Additionally, the successful creation of a functioning plasmid shows that this strategy is feasible for the production of plant-based vaccines. To further confirm and apply this approach in practical vaccination applications, future research should concentrate on large-scale production, in vivo efficacy testing, and direct plant transformation.

F. SUPPLEMENTARY MATERIAL

Suppelementary Material: Rational Design of Dengue Vaccines: Integrating Mutagenesis, Structure Prediction, and Docking Simulations in Nicotiana tabacum

G. REFERENCES

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