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Cloning of a Rift Valley Fever fusion gene in a plant virus derived replicon vector

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ABSTRACT

Rift Valley fever (RVF) disease is an emerging viral zoonotic disease caused by Rift Valley fever virus (RVFV). RVF disease is seemingly becoming more severe in endemic populations and in areas of new outbreak. RVF disease is listed by WHO as requiring urgent research and development attention. There is no therapeutics or licensed vaccine for human use in the case of an eventual outbreak. There is a need to clone RVFV genes in cloning vectors which may be useful to produce antigens in plant cells or may be tested directly as DNA vaccines. A synthesized Rift Valley fever virus fusion gene was cloned in a bean yellow dwarf virus derived replicon vector; PBYR2e3K2Mc-GFP by removing and replacing the GFP gene. The cloned PBYR2e3K2Mc-RVFV fusion gene was confirmed by PCR, restriction digestion and DNA sequencing. This cloned RVFV fusion gene in plant virus vector can be used for subsequent protein expression in plants cells or used directly tested as DNA vaccines in future studies.

***Keywords*:** DNA Cloning, RVFV fusion gene, bean yellow dwarf derived vector, RVF disease, zoonotic disease, PBYR2e3K2Mc-RVFV, geminivirus vector, *Escherichia coli* DH5α cells, emerging disease, PBYR2e3K2Mc-GFP

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1. INTRODUCTION

The Rift valley fever virus (RVFV), belonging to the family *Bunyaviridae*, genus *Phlebovirus* is responsible for Rift valley fever (RVF) disease, an arthropod-borne zoonotic disease affecting majorly livestock. RVF is highly contagious and infectious bites of particularly *Aedes* and *Culex* mosquitoes can easily transmit the virus between humans and animals, humans also get infected when they come in contact with infectious material such as blood or body fluids or consume infected animal products. The disease is reported to have significant economic impact on farmer's livelihood due to the high mortality in livestock, and also causes severe disease in humans involving hospitalizations and death (1-5). Since the discovery of RVF in Kenya in 1931 (6), the disease has spread through Africa and also to the Arab peninsula resulting in over a thousand reported human deaths (7-9). Despite these reports, lack of systematic surveillance to determine the actual incidence of the disease and low alarming death ratio compared to that of COVID-19, tuberculosis and malaria still pose challenges in developing vaccines and drugs to combat the disease. However, Rift valley disease (RVF) is still on the list of World Health Organization (WHO) as a blueprint priority disease requiring urgent research attention. Despite the absence of any official report of outbreaks of RVF in countries such as Nigeria, studies conducted as far back as four decades ago and even recent publications suggest the presence of antibodies to the virus in livestock and humans (10-13).

Thus, there is an urgent need to develop vaccines and diagnostic against the disease (9). Molecular cloning has become a reliable standard in recombinant DNA technology. It is an essential first step towards producing large quantities of recombinant protein in cells (14-16). The process can be used to produce very essential therapeutics and antigens (17) or applied directly as plasmid vaccines (18-20). Bacteria are essential model organisms used in cloning for amplification of cloned DNA (21). The bean yellow dwarf virus (BeYDV)-derived replicon vector; PBYR2e3K2Mc-GFP is a unique geminivirus derived vector with the ability to replicate in high numbers in cells (22). Some reports have demonstrated the application of this geminivirus vector for protein expression studies and the production of recombinant protein of interest (23-25).

In this study, we demonstrate for the first-time successful cloning of epitopes of the viral envelope proteins (Gn and Gc) segment from RVFV strain ZH548 in a plant virus vector PBYR2e3K2Mc-GFP. Analysis of the DNA by PCR and electrophoresis confirm the size and orientation of the sequence to be 1574 bp. The success of this clone in bacterial system will help further investigate other experimental studies including RVFV protein expression in *E. coli* and vaccine development.

2. MATERIALS AND METHOD

2. 1. Design of RVFV Fusion Gene constructs and Bacterial Strain

The Gc and Gn RVFV fusion gene construct was designed by joining dominant and subdominant epitopes of Gn and Gc of M segment of RVFV strain ZH548 with linkers. The construct was designed to contain sequence of Gn epitope (811 bp) at the 5' end joined by linker1 (47 bp) with Gc1 sequence (291 bp). Gc1 and Gc (314 bp) were fused by joining with linkers2 (45bp) at the 3'end of the construct (Figure 1a).

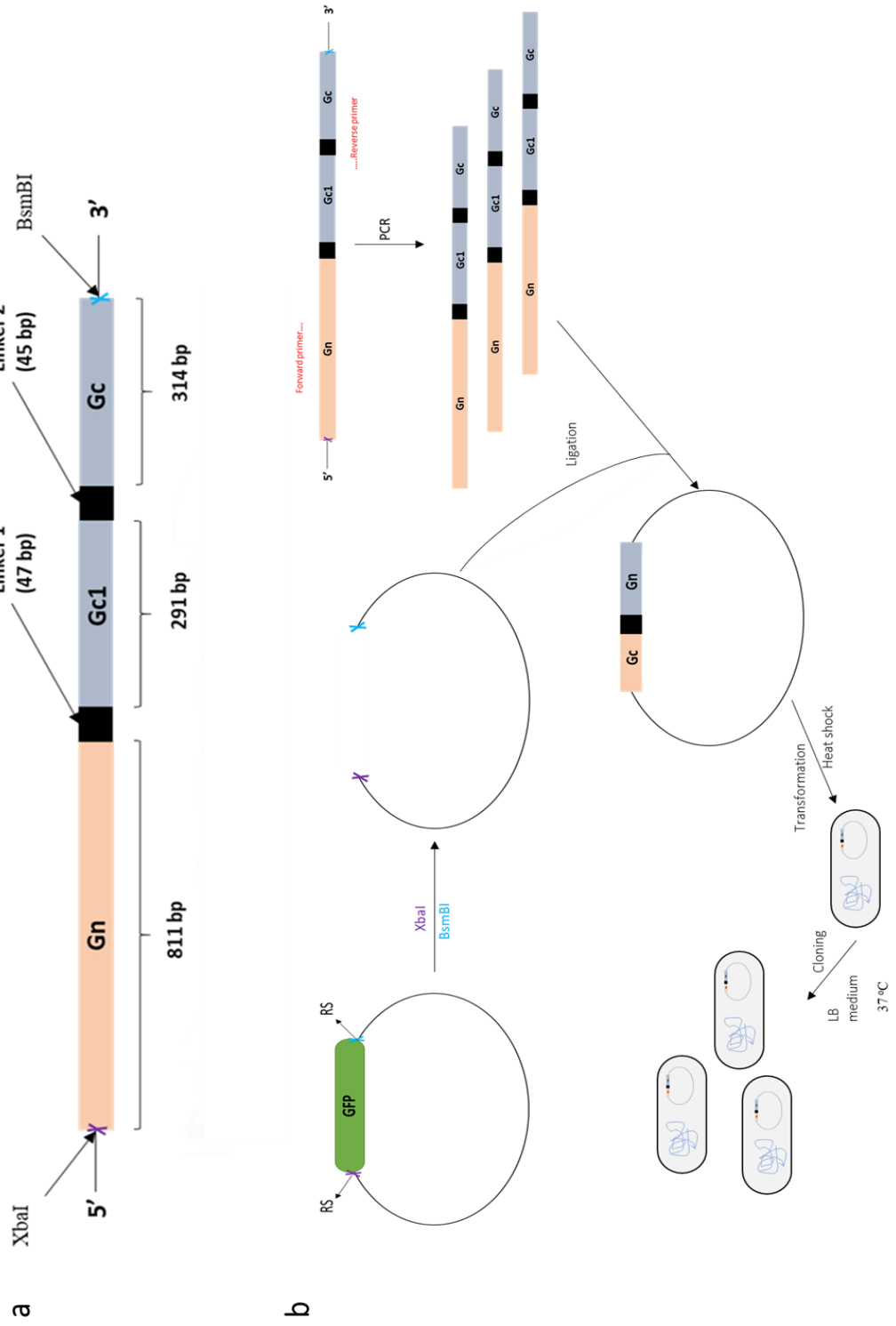

Figure 1. Cloning of RVFV fusion gene into a plant virus derived PBYR2e3K2Mc-GFP vector. 1a. Gene construct of RVFV fusion gene 1b. shows cloning of the synthesized RVFV fusion gene in plant virus derived PBYR2e3K2Mc-GFP vector by removal of the GFP gene and its transformation into *E.coli* DH5α in LB medium supplemented with kanamycin

The constructs were also designed to include restriction sites for XbaI and BsmBI (NEB, USA) respectively at 5' and 3' ends and synthesized commercially by GeneScript, Inc, USA.

BeYDV-derived replicon vector; PBYR2e3K2Mc-GFP (21) was provided by Dr Hugh Mason, of the Center for Infectious Diseases and Vaccinology, Biodesign Institute and School of Life Sciences; Arizona State University; Tempe, AZ USA.

The commercial bacterial strain of *E. coli* DH5α was routinely cultured in Luria-Bertani (LB) medium or on LB agar supplemented with 10 mg/L kanamycin for amplification of cloned genes. The growth condition was set at 37 °C for every duration.

2. 2. Polymerase Chain Reaction

PCR was used for the amplification of the RVFV fusion construct using forward primer – 5'-CAGCGCCGACATCTAGAACATATGATTTCAGGCTCAAATA-3' and reverse primer reverse primer – 3'GCTCGGGCCGTCTCAGTACCGGGTTAATGATGATGATGA TGATGATATGAGACAATCAATAAT-5'. The forward and reverse primers were designed with XbaI restriction site and BsmBI/BsrG1 restriction sites, respectively.

The RVFV fusion gene was amplified with insert specific primers using Q5 high-fidelity DNA polymerase (New England Biolabs (NEB), USA). A 50 µL PCR reaction was set up. PCR condition were as follows; Initial denaturation condition of 98 °C for 2 minutes, then denaturation at 98 °C for 20 seconds, with gradient annealing temperature ranging from 48 °C to 65 °C for 45 seconds, extension at 72 °C for 1minute and a final extension of 72 °C for 5minutes for 30 cycles using a gradient PCR machine (BioRad, USA).

PCR product was separated on 1% agarose gel containing ethidium bromide, bands were visualised with a gel documentation system. PCR amplified product was purified with PCR purification kit (Qiagen, Germany) and quantified with nanodrop (ThermoFisher Scientific, USA).

2. 3. Restriction digestion of RVFV fusion gene insert and PBYR2e3K2Mc Plasmid, and ligation

Restriction digestion was carried out on PCR amplified RVFV fusion gene with XbaI and BsmBI (NEB, USA). PBYR2e3K2Mc-GFP was also digested with both XbaI and BSrGI (NEB, USA) and subsequently treated with antartic phosphatase enzyme (NEB, USA). Both restriction digested insert and vector were purified with nucleotide purification kit (Qiagen, Germany) and concentration determined with nanodrop (ThermoFisher Scientific, USA). PBYR2e3K2Mc vector was mixed with digested RVFV-fusion gene with T4 DNA ligase by overnight ligation at 16 °C.

2. 4. Transformation of competent *E. coli* DH5α cells with PBYR2e3K2Mc-RVFV fusion gene plasmid

Ligated PBYR2e3K2Mc-RVFV was transformed into competent *E. coli* DH5α cells by heat shock method and immediately plated on LB agar supplemented with kanamycin and incubated overnight at 37 °C.

Transformed colonies were screened by PCR using insert specific primers, plasmid were also isolated and also screened for insert of interest by PCR using insert specific primers, as well as restriction digestion of plasmid with XbaI and BsmBI (New England Biolabs, USA) to ascertain clones and also by sequencing of genes.

3. RESULTS AND DISCUSSION

3. 1. Cloning of RVFV fusion gene

Cloning of DNA is an inevitable step in making heterologous protein in large quantities in cells (26). Cloning has been used to produce recombinant protein which have many applications including their use as vaccines and therapeutics (15, 17, 27). A synthesized RVFV fusion gene was cloned into a plant viral vector; PBYR2e3K2Mc-GFP by removing GFP with the restriction enzymes XbaI and BSRGI. PCR conditions for the synthesized RVFV fusion gene was optimized after running series of gradient PCR reactions. The optimal annealing temperature determined from gradient PCR was 50 °C for 50 seconds. The expected band size for the RVFV fusion gene after PCR is 1574bp which corresponded to the size of PCR amplicon on viewing in 1% agarose as presented in Figure 2. This annealing temperature condition was used for all subsequent PCR reactions.

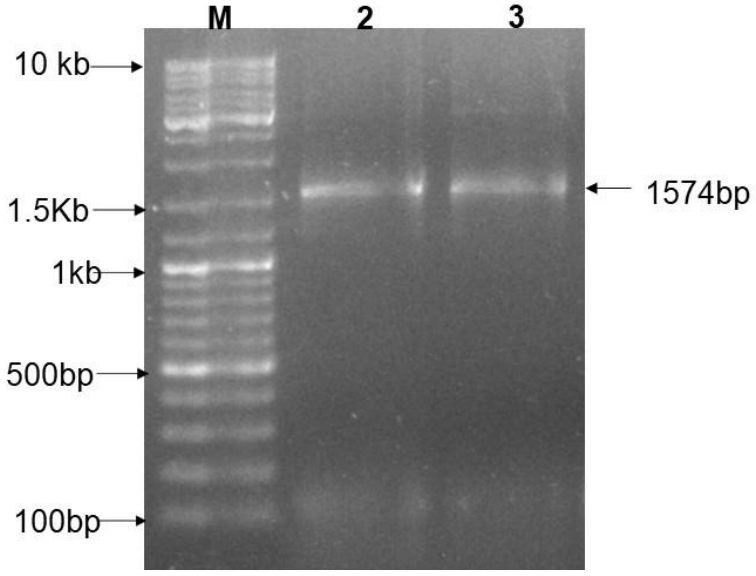

Figure 2. PCR amplified RVFV fusion gene, Lane M; 10kb ladder mix (Thermofischer Scientific, USA), Lane 2 and Lane 3; amplified RVFV fusion gene insert (expected band size 1,574bp)

3. 2. Cloning of RVFV fusion gene in PBYR2e3K2Mc-GFP replicon vector

Restriction digestion of PBYR2e3K2Mc-GFP replicon vector with XbaI and BSRGI and RVFV fusion gene with XbaI and BSMBI was carried out to allow compatible ends for both genes for cloning. Following successful cloning of PBYR2e3K2Mc-RVFV fusion gene, cloned gene was transformed in *E. coli* DH5α cells, and transformants first confirmed with colony PCR and subsequently plasmid was isolated and again confirmed by PCR using gene specific primers. PCR amplified RVFV fusion gene was digested with both XbaI and BSMBI to allow compatible ends for both genes for cloning. Both vector and insert where ligated together to produce PBYR2e3K2Mc-RVFV plasmid, after which several methods were used to verify that RVFV fusion gene insert had successfully cloned into the vector. Positive clones were selected

on LB agar containing 50 mg/L kanamycin, this was followed by colony PCR and PCR on isolated plasmid DNA using insert specific primer confirming the presence of our gene interest (Figure 3 and 4). Isolated plasmid was also subjected to restriction mapping and restriction enzyme digestion (Figure 5) which is a very precise method of ascertaining the insert successfully cloned.

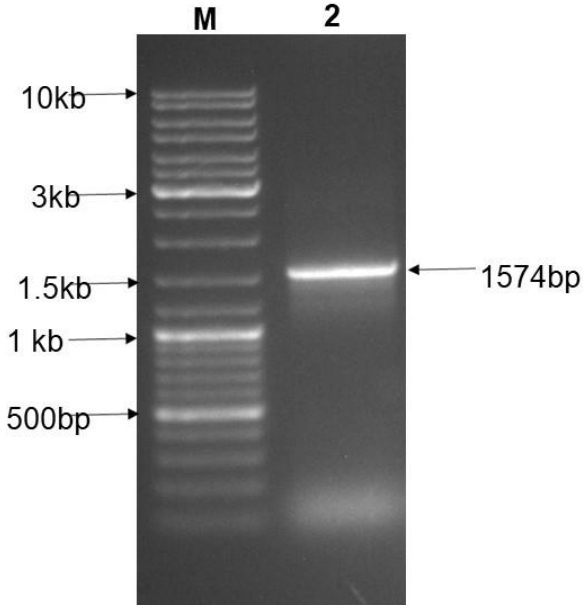

Figure 3. Colony PCR confirmation of RVFV fusion gene in PBYR2e3K2Mc replicon vector with insert specific primers, Lane M; 10kb ladder mix (Thermofischer Scientific, USA), Lane 2; Amplified RVFV fusion gene (expected band size 1574bp)

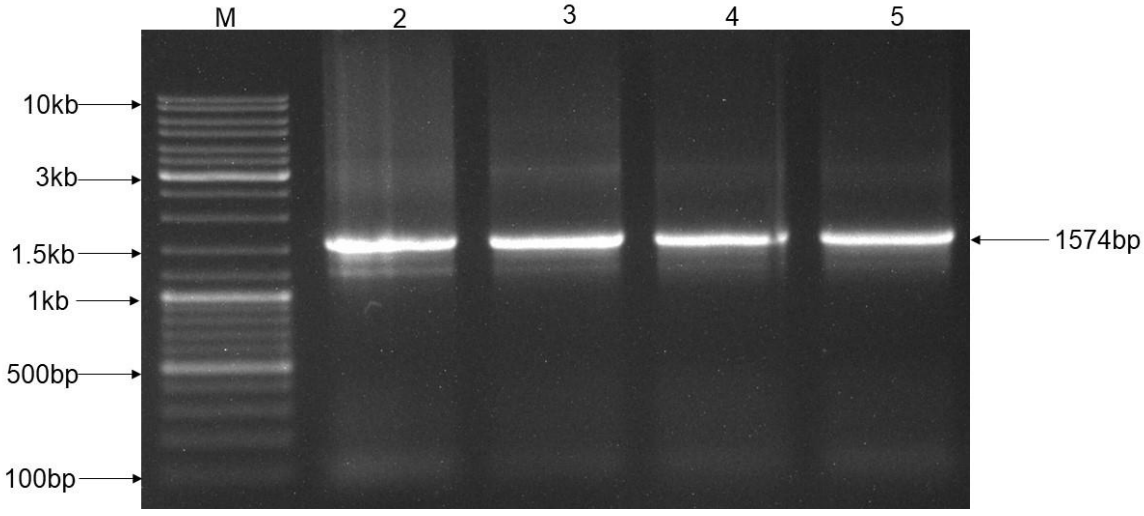

Figure 4. PCR confirmation of PBYR2e3K2Mc-RVFV plasmid isolated from *E. coli* DH5α cells. Lane M; 10kb ladder mix (Thermofischer Scientific, USA), Lane 2; Positive control, Lane 3-5; Amplified RVFV fusion gene (expected band size 1,574bp)

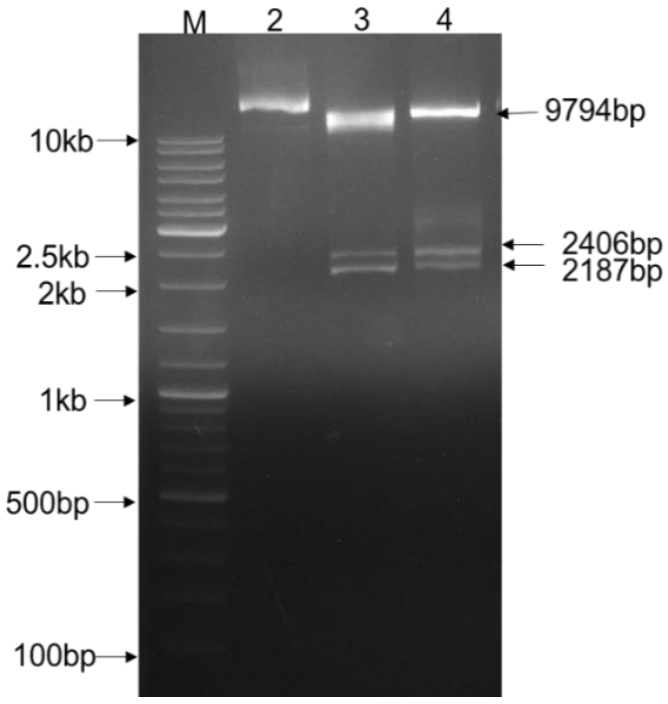

Figure 5. Confirmation of PBYR2e3K2Mc- RVFV plasmid by restriction digestion with EcoRI and BSRGI. Lane M; 10kb ladder mix (Thermofischer Scientific, USA), Lane 2; undigested plasmid, Lane 3-4: Digested PBYR2e3K2Mc- RVFV fusion gene plasmid (expected band sizes are 9794bp, 2406bp and 2187bp)

3. 3. Sequenced RVFV fusion gene

Most specifically Sanger sequencing across the insert only was used to confirm the sequence of the RVFV fusion gene, sequenced insert showed a 99.4% similarity in comparison with the synthesized RVFV insert, which is acceptable given the limitations of Sanger sequencing (28). This study accomplished the first step of cloning the RVFV fusion gene into a plant viral derived replicon vector.

The sequenced RVFV fusion gene has a 99.4% similarity with the synthesized RVFV fusion gene as shown below.

Length: 1425
Identity: 1417/1425 (99.4%)
Similarity: 1417/1425 (99.4%)
Gaps: 4/1425 (0.3%)
Score: 7029.0

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Synthesized RVFV 3428 TCATTGAGAGCCCA-GGCAA-AGGGTATGCAATTGTTGATGAGCCATTCT

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Sequenced RVFV       1 TCGTTGAGAGCCCACAGCAAGAGGTAATGCAATTGTTGATGAGCCATTCT
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Synthesized RVFV 3476 CAGAAATT-CCTCGGCAAGGGTTCTTGGGGGAGATCAGGTGCAATTCAGA
                      |||||||| ||||||||||||||||||||||||||||||||||||||||
Sequenced RVFV   51   CAGAAATTACCTCGGCAAGGGTTCTTGGGGGAGATCAGGTGCAATTCAGA

Synthesized RVFV 3525G TCCTCAGTCCTGAGTGCTCATGAATCATGCCTTAGGGCACCAAACCTTA
                       ||||||||||||||||||||||||||||||||||||||||||||||||||
Sequenced RVFV   101   GTCCTCAGTCCTGAGTGCTCATGAATCATGCCTTAGGGCACCAAACCTTA

Synthesized RVFV 3575  TCTCATACAAGCCCATGATAGATCAATTGGAGTGCACAACAAATCTGATT
                       ||||||||||||||||||||||||||||||||||||||||||||||||||
Sequenced RVFV   151   TCTCATACAAGCCCATGATAGATCAATTGGAGTGCACAACAAATCTGATT

Synthesized RVFV3625 GATCCCTTTGTTGTCTTTGAGAGGGGTTCTCTGCCACAGACAAGGAATGA
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Sequenced RVFV   201 GATCCCTTTGTTGTCTTTGAGAGGGGTTCTCTGCCACAGACAAGGAATGA

Synthesized RVFV 3675 CAAAACCTTTGCAGCTTCAAAAGGAAATAGAGGTGTTCAAGCTTTCTCTA
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
Sequenced RVFV   251  CAAAACCTTTGCAGCTTCAAAAGGAAATAGAGGTGTTCAAGCTTTCTCTA

Synthesized RVFV 3725 AGGGCTCTGTACAAGCTGATCTAACTCTGATGTTTGACAATTTTGAGGTG
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
Sequenced RVFV   301  AGGGCTCTGTACAAGCTGATCTAACTCTGATGTTTGACAATTTTGAGGTG

Synthesized RVFV 3775 GACTTTGTGGGAGCAGCCGTATCTTGTGATGCCGCCTTCTTAAATTTGAC
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
Sequenced RVFV   351  GACTTTGTGGGAGCAGCCGTATCTTGTGATGCCGCCTTCTTAAATTTGAC
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Synthesized RVFV 3825 AGGTTGCTATTCTTGCAATGCAGGGGCCAGGGTCTGCCTGTCTATCACAT
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Sequenced RVFV   401  AGGTTGCTATTCTTGCAATGCAGGGGCCAGGGTCTGCCTGTCTATCACAT

Synthesized RVFV 3875 CCACAGGAACTGGATCTCTCTCTGCCCACAATAAGGATGGGTCTCTGCAT
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Sequenced RVFV   451  CCACAGGAACTGGATCTCTCTCTGCCCACAATAAGGATGGGTCTCTGCAT

Synthesized RVFV 3925 ATAGTCCTTCCATCAGAGAATGGAACAAAAGACCAGTGTCAGATACTACA
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Sequenced RVFV   501  ATAGTCCTTCCATCAGAGAATGGAACAAAAGACCAGTGTCAGATACTACA

Synthesized RVFV 3975 CTTCACTGTGCCTGAAGTAGAGGAGGAGTTTATGTACTCTTGTGATGGAG
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Sequenced RVFV   551  CTTCACTGTGCCTGAAGTAGAGGAGGAGTTTATGTACTCTTGTGATGGAG

Synthesized RVFV4025 ATGAGCGGCCTCTGTTGGTGAAGGGGACCCTGATAGCCATTGATCCATTT
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
Sequenced RVFV 601   ATGAGCGGCCTCTGTTGGTGAAGGGGACCCTGATAGCCATTGATCCATTT

Synthesized RVFV 4075 GATGATAGGCGGGAAGCAGGGGGGGAATCAACAGTTGTGAATCCAAAATC
                     |||||||||||||||| ||||||||||||||||||||||||||||||||
Sequenced RVFV   651  GATGATAGGCGGGAAGCA-GGGGGGAATCAACAGTTGTGAATCCAAAATC

Synthesized RVFV 4125 TGGATCTTGGAATTTCTTTGACTGGTTTTCTGGACTCATGAGTTGGTTTG
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Sequenced RVFV   700  TGGATCTTGGAATTTCTTTGACTGGTTTTCTGGACTCATGAGTTGGTTTG
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Synthesized RVFV 4175 GAGGGCCTCTTAAAACTATACTCCTCATTTGCCTGTATGGCGGCGGCGGC
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Sequenced RVFV   750  GAGGGCCTCTTAAAACTATACTCCTCATTTGCCTGTATGGCGGCGGCGGC

Synthesized RVFV 4225 AGCGGCGGCGGCGGCAGCGGCGGCGGCGGCAGCATGCACTGTCCAAATGA
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Sequenced RVFV    800 AGCGGCGGCGGCGGCAGCGGCGGCGGCGGCAGCATGCACTGTCCAAATGA

Synthesized RVFV 4275 CTACCAGTCAGCTCATTACCTCAACAATGACGGGAAAATGGCTTCAGTCA
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Sequenced RVFV   850  CTACCAGTCAGCTCATTACCTCAACAATGACGGGAAAATGGCTTCAGTCA

Synthesized RVFV 4325 AGTGCCCTCCTAAGTATGAGCTCACTGAGGACTGCAACTTTTGTAGGCAG
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Sequenced RVFV   900  AGTGCCCTCCTAAGTATGAGCTCACTGAGGACTGCAACTTTTGTAGGCAG

Synthesized RVFV 4375 ATGACAGGTGCTAGCCTGAAGAAGGGGTCTTATCCTCTCCAAGACTTGTT
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Sequenced RVFV   950  ATGACAGGTGCTAGCCTGAAGAAGGGGTCTTATCCTCTCCAAGACTTGTT

Synthesized RVFV 4425 TTGTCAGTCAAGTGAGGATGATGGATCAAAATTAAAAACAAAAATGAAAG
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Sequenced RVFV   1000 TTGTCAGTCAAGTGAGGATGATGGATCAAAATTAAAAACAAAAATGAAAG

Synthesized RVFV 4475 GGGTCTGCGAAGTGGGGGTTCAAGCACTCAAAAAGTGTGATGGCCAACTC
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Sequenced RVFV   1050 GGGTCTGCGAAGTGGGGGTTCAAGCACTCAAAAAGTGTGATGGCCAACTC
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Synthesized RVFV 4525 AGCACTGCACATGAGGTTGTGCCCTTTGGCGGCGGCGGCAGCGGCGGCGG
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
Sequenced RVFV   1100 AGCACTGCACATGAGGTTGTGCCCTTTGGCGGCGGCGGCAGCGGCGGCGG

Synthesized RVFV 4575 CGGCAGCGGCGGCGGCGGCAGCATGGCCTTTGTTGTTGTGTTTGTATTCA
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
Sequenced RVFV   1150 CGGCAGCGGCGGCGGCGGCAGCATGGCCTTTGTTGTTGTGTTTGTATTCA

Synthesized RVFV 4625 GTTCTATTGCAATAATTTGTTTAGCTATTCTTTATAGGGTGCTTAAGTGC
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
Sequenced RVFV   1200 GTTCTATTGCAATAATTTGTTTAGCTATTCTTTATAGGGTGCTTAAGTGC

Synthesized RVFV 4675 CTGAAGATTGCCCCAAGGAAAGTTCTGAATCCACTAATGTGGATCACAGC
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
Sequenced RVFV   1250 CTGAAGATTGCCCCAAGGAAAGTTCTGAATCCACTAATGTGGATCACAGC

Synthesized RVFV 4725 CTTCATCAGATGGATATATAAGAAGATGGTTGCCAGAGTGGCAGACAACA
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
Sequenced RVFV   1300 CTTCATCAGATGGATATATAAGAAGATGGTTGCCAGAGTGGCAGACAACA

Synthesized RVFV  4775 TTAATCAAGTGAACAGGGAAATAGGATGGATGGAAGGAGGTCAGTTGGTT
                       ||||||||||||||||||||||||||||||||||||||||||||||||||
Sequenced RVFV    1350 TTAATCAAGTGAACAGGGAAATAGGATGGATGGAAGGAGGTCAGTTGGTT

Synthesized RVFV  4825 CTAGGGAACCCTGCCCCTATTCCTC   4849
                       |||||||||||||||||||||||||
Sequenced RVFV    1400 CTAGGGAACCCTGCCCCTATTCCTC   1424
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4. CONCLUSION

This study successfully cloned the RVFV fusion gene in a bean yellow dwarf virus replicon vector PBYR2e3K2Mc-RVFV. This will be used in future studies to produce antigens and as DNA vaccines that will be tested as a potential vaccine candidate.

Acknowledgement

The authors wish to thank Dr Hugh Mason, of the Center for Infectious Diseases and Vaccinology, Biodesign Institute and School of Life Sciences; Arizona State University; Tempe, AZ USA for providing the BeYDV-derived replicon vector; PBYR2e3K2Mc-GFP. The authors also wish to thank Department of Biotechnology – The World Academy of Sciences (DBT-TWAS) for funding the project and The Department of Biotechnology, Bharathiar University for providing the facilities and equipment to carry out the research work.

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