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Development of Taï Forest alphavirus trans-replicase assay systems in mammalian and insect cells

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Development of Taï Forest alphavirus trans-replicase assay systems in mammalian and insect cells

Taï Forest alphavirus (TALV) is a poorly characterised insect-specific alphavirus that has remained difficult to study since its discovery due to its inability to grow in standard cell cultures. This study investigates the TALV by developing a mammalian and insect cell-based trans-replicase assay system. To develop the assay, plasmids separating viral replication into two functional components, the replication driver (replicase) and the replication template (minigenome), were constructed and tested by transfection into respective cell lines. The trans-replicase and split trans-replicase systems were used to investigate the compatibility of the TALV minigenome with replicases of other alphaviruses and *vice versa*. These results offer the first functional analysis of TALV replication, revealing that it is temperature-sensitive and dependent on unknown factors. This insight establishes a baseline for future studies of this understudied alphavirus.

Keywords: alphavirus, Taï Forest alphavirus, trans-replicase assay, replicase, minigenome
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Institute name: Institute of Bioengineering, Laboratory of Applied Virology

Taï Forest alfaviiruse trans-replikaasi testsüsteemide arendamine imetaja- ja putukarakkudes

Taï Forest alfaviirus (TALV) on väheuuritud putukaspetsiifiline alfaviirus, mille uurimist on alates selle avastamisest raskendanud viiruse võimetus paljuneda tavapärastes rakukultuurides. Käesolevas töös uuriti TALV-e trans-replikaasi testsüsteemide abil imetaja- ja putukarakkudes. Selleks disainiti ja konstrueeriti vastavad replikaasi- ja minigenoomiplasmiidid ning hinnati nende funktsionaalsust transfektsioonikatsetes erinevates rakuliinides. TALV-i minigenoomi ja teiste alfaviiruste replikaaside vastastikuse ühilduvuse analüüsimiseks kasutati nii *trans*-replikaasi kui ka *split-trans*-replikaasi süsteeme. Tulemused näitasid, et TALV-i replikatsioon on temperatuuritundlik ning sõltub seni tuvastamata faktoritest. Käesolev töö annab esimese funktsionaalse ülevaate TALV-i replikatsioonist ning loob aluse selle väheuuritud alfaviiruse edasiseks uurimiseks.

Võtmesõnad: alfaviirus, Taï Forest alfaviirus, *trans*-replikaasi süsteem, replikaas, minigenoom

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TERMS, ABBREVIATIONS AND NOTATIONS

AlbPolI	*Aedes albopictus* RNA polymerase I
BFV	Barmah forest virus
CHIKV	Chikungunya virus
CMV	cytomegalovirus
CSE	conserved sequence element
EEEV	Eastern equine encephalitis virus
EILV	Eilat virus
FLuc	*Firefly* luciferase
GAA	polymerase-inactive mutation (Glycine-Alanine-Alanine)
GDD	catalytic motif (Glycine-Aspartic acid-Aspartic acid)
GLuc	*Gaussia* luciferase
HSPolI	*Homo sapiens* RNA polymerase I
MAYV	Mayaro virus
ns	non-structural
nsP	non-structural protein
ONNV	O'nyong'nyong virus
ORF	open reading frame
poly-A	polyadenylation sequence
PS	packaging signal
RC	replication complex
RLU	relative light unit
RNA	ribonucleic acid
RRV	Ross River virus
RSE	repeated sequence element
SFV	Semliki Forest virus
SG	sub-genomic
SINV	Sindbis virus
TALV	Taï Forest alphavirus
Ubi	*Aedes aegypti* polyubiquitin
UTR	untranslated region
VEEV	Venezuelan equine encephalitis virus
WEEV	Western equine encephalitis virus
wt	wild type

INTRODUCTION

Alphaviruses are a genus of RNA viruses in the family *Togaviridae*, primarily transmitted by mosquitoes. Their genome consists of positive-sense single-stranded RNA and encodes proteins required for viral RNA replication and viral particle formation. Several alphavirus species can infect both mosquito and vertebrate hosts, including humans, and are therefore important public health pathogens.

This study focuses on the recently discovered alphavirus Taï Forest alphavirus (TALV). In comparison with better-characterised alphaviruses, relatively little is known about TALV, and the experimental conditions required for its study have not yet been fully optimised. Moreover, direct investigation of viral replication using infectious virus would require higher biosafety containment and could complicate the experimental setup. For these reasons, an alternative experimental approach was adopted.

The trans-replicase assay is a valuable tool for studying novel viruses in a controlled environment. This allows studying the viral RNA replication without generating infectious virus particles and interchange of replication components derived from various alphaviruses, which evaluates their functional compatibility.

The aim of this thesis was to develop the TALV trans-replicase assay systems in mammalian and insect cells to evaluate their replication activity in different cell lines and investigate the functional compatibility of TALV replication components with those of other alphaviruses.

I want to thank Sandra Koit and Andres Merits for their supervision and valuable help. I am also grateful to Eva Žusinaite for guidance and support.

I Literature review

1.1 ALPHAVIRUSES

Alphaviruses are a genus of positive-strand RNA viruses within the *Togaviridae* family, consisting of 32 identified viruses (Ahola *et al.*, 2021). They infect a range of vertebrate and invertebrate hosts and are mostly transmitted by mosquitoes (Ahola *et al.*, 2021). Based on the diseases they cause in humans, alphaviruses are commonly classified as arthritogenic or encephalitic (Strauss & Strauss, 1994).

Arthritogenic alphaviruses primarily cause febrile illness and joint pain that can become chronic. Chikungunya virus (CHIKV) is the most medically important of these alphaviruses because it can cause large epidemics and persistent arthritis-like disease (Strauss & Strauss, 1994). First isolated in Tanzania in 1952 (Carey, 1971), CHIKV was historically confined to Africa, Asia, and the Indian Ocean region, but since 2004, it has spread much more widely. Other arthritogenic alphaviruses are generally more localised or less severe. For example, O'nyong-nyong (ONNV) virus is restricted to Sub-Saharan Africa and transmitted by *Anopheles* mosquitoes (Williams *et al.*, 1965). In Latin America, Mayaro virus (MAYV) is considered a potential risk for urban outbreaks (Caicedo *et al.*, 2021). Meanwhile, Ross River virus (RRV) and Barmah Forest virus (BFV) remain isolated to Australia (Jacups *et al.*, 2008). The remaining species, such as Semliki Forest virus (SFV) and the broadly distributed Sindbis virus (SINV), cause a relatively mild illness (Public Health Agency of Canada, 2021; MacLachlan & Dubovi, 2017).

Unlike the arthritogenic alphaviruses, encephalitic alphaviruses are much more dangerous and trigger severe neurological diseases. These dangerous species, specifically Venezuelan, Eastern and Western equine encephalitis viruses (VEEV, EEEV, WEEV), are primarily distributed across the Americas (Griffin, 2008).

Finally, Eilat virus (EILV) and TALV are unique among alphaviruses. They are insect-specific viruses that replicate only in mosquitoes and cannot replicate in vertebrate cells (Nasar *et al.*, 2012; Hermanns *et al.*, 2017).

1.2 PHYLOGENY OF ALPHAVIRUSES

To understand the evolutionary relationships among alphaviruses, it is essential to consider their classification. Based on geographic distribution, alphaviruses are broadly divided into Old World and New World groups (Azar *et al.*, 2020). Old World alphaviruses are distributed across Eurasia and Africa and include CHIKV, ONNV, SINV, and RRV (among others) (Azar *et al.*, 2020). Conversely, New World alphaviruses are found in the Americas and include VEEV, EEEV, and WEEV (Azar *et al.*, 2020).

Beyond geographic distribution, alphaviruses can also be grouped into complexes based on clinical, antigenic, and genetic characteristics. Antigenic classification relies on immune cross-reactivity, whereby conserved epitopes on the viral surface glycoproteins (E1 and E2) are recognised by antibodies (Hatami *et al.*, 2026), a method historically used to classify alphaviruses (Weaver *et al.*, 2012). Genetically, alphaviruses are classified into clades based on capsid structural regions, non-structural coding regions and complete viral genomes (Luers *et al.*, 2005). Nowadays, these complementary approaches have enabled the definition of alphavirus complexes and the reconstruction of their phylogenetic relationships (Figure 1).

TALV and EILV belong to an unidentified complex due to their unique host-specificity. TALV remains one of the least characterised members of the *Alphavirus* genus (Bell *et al.*, 2024).

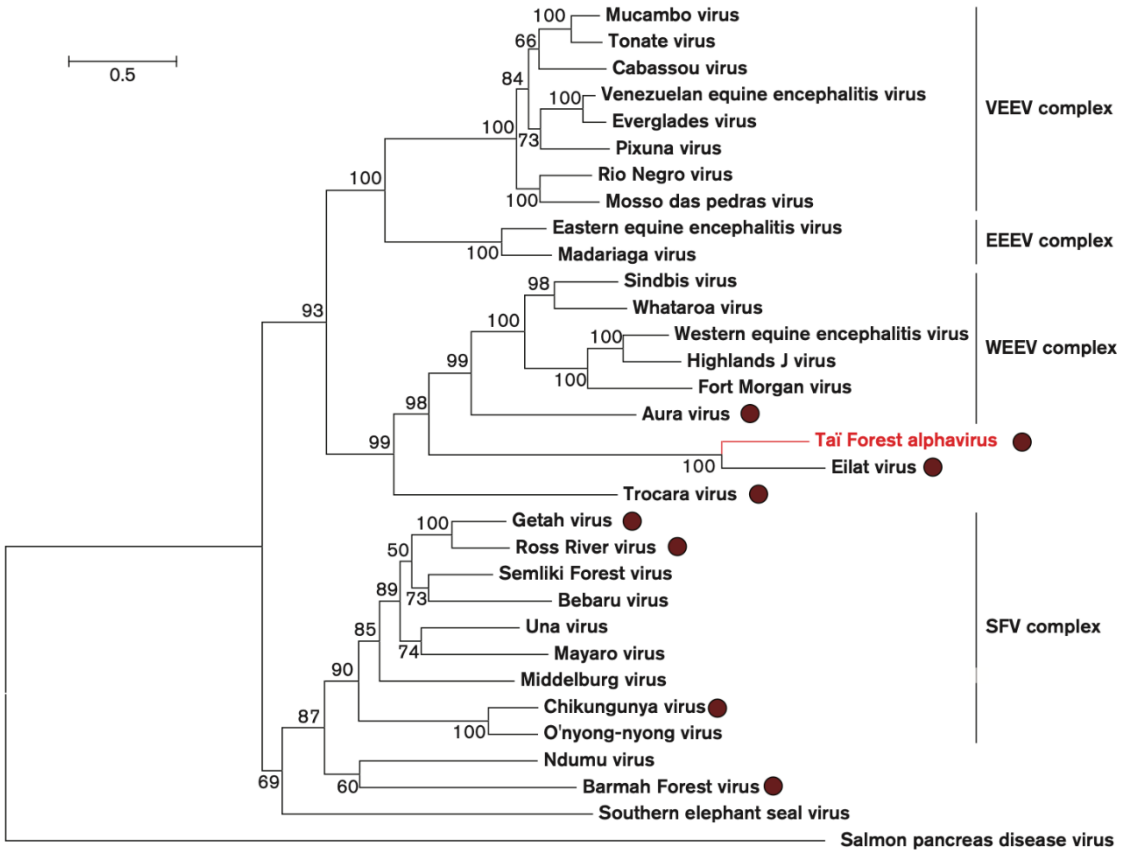

Figure 1. Phylogenetic tree of alphaviruses. The tree reconstruction is based on the structural protein open reading frame (ORF). Position of TALV is highlighted in red. Circles marked with brown represent the viruses with RRV-type RSEs in the 3'-NTR (Hermanns *et al.*, 2017).

1.3 ALPHAVIRUS GENOME

The alphavirus genus is characterised by a positive-sense, single-stranded RNA genome approximately 11.5-12 kilobase in length (Ahola *et al.*, 2021). The genome is organised into coding regions and non-coding regions, which are flanked by 5' cap-0 and 3' poly-A tail that allows the viral genome to mimic host mRNA (Strauss & Strauss, 1994).

The coding region contains two open reading frames (ORFs) (Ahola *et al.*, 2021). The first ORF encodes for non-structural proteins (nsP1-nsP4), which, after further processing, form the replication complex (RC) responsible for RNA synthesis (Figure 2). The second ORF encodes the structural proteins, including envelope glycoproteins (E1-E3), capsid protein (CP), and 6K protein (or transframe protein), which are required for virion assembly (Leung *et al.*, 2011).

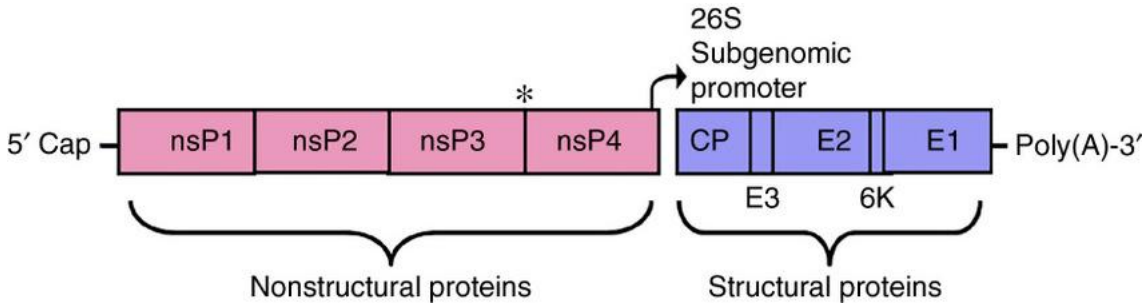

Figure 2. Scheme of alphaviral genome structure. At the 5' end, genomic RNA possesses a cap-0 structure, named 5'-Cap. The RNA molecule encodes non-structural protein sequences (nsP1-nsP4) and structural protein sequences (CP-E3-E2-6K-E1). The 3' end possesses a poly-A tail, which is referred to as Poly(A)-3' (Tomar & Narwal, 2017). * Denotes the leaky codon which regulates the production of nsP4.

1.3.1 Non-coding regions

The genome contains non-coding regions, such as the 5' untranslated region (5'-UTR), intergenic region and the 3' untranslated region (3'-UTR) (Figure 3) (Ahola *et al.*, 2021). These regions harbour *cis*-acting RNA elements that play essential roles in regulating viral replication (Rupp *et al.*, 2015). *Cis*-acting elements are typically located at the boundaries of coding sequences, although some may also occur within coding regions. Among the most important *cis*-acting elements are the four conserved sequence elements (CSE1-4) (Strauss & Strauss, 1994).

The 5'-UTR is located upstream of the first ORF. It contains CSE1 that initiates negative-strand RNA synthesis and acts as a promoter of positive-strand RNA synthesis (Figure 3) (Ahola *et al.*, 2021). Downstream of the 5'-UTR, within the sequence encoding nsP1, CSE2 enhances the synthesis of both positive- and negative-strand RNAs (Figure 3) (Frolov *et al.*, 2001). The third CSE, also known as CSE3 or the SG promoter, is present in the intergenic region and partially overlaps the nsP4 coding region (Figure 3) (Bell *et al.*, 2024). The SG promoter is required for the initiation of SG RNA synthesis. At 3' UTR, CSE4 functions as a core promoter for the initiation of negative-strand RNA synthesis (Figure 3) (Luo *et al.*, 2025).

However, CSEs are not the only features required for efficient alphaviral replication and transcription. For example, the packaging signal (PS) is a *cis*-acting RNA element that directs the selective encapsidation of genomic RNA into viral particles (Ahola *et al.*, 2021). Its genomic location varies among alphaviruses (Rupp *et al.*, 2015). For example, in several New World alphaviruses, the PS is located within the nsP1 coding region, whereas in certain Old World alphaviruses, it has been mapped to the nsP2 region (Figure 3) (Mendes & Kuhn, 2018).

At the 3’ end of the genome, the 3’-UTR represents the longest non-coding region due to the presence of repeated sequence elements (RSEs) (Figure 3) (Rupp *et al.*, 2015). RSEs are non-conserved motifs that vary between alphaviral species and are primarily involved in host-specific adaptation (Rupp *et al.*, 2015). For example, the TALV RSEs are critical for replication in insect cells (Hermanns *et al.*, 2017).

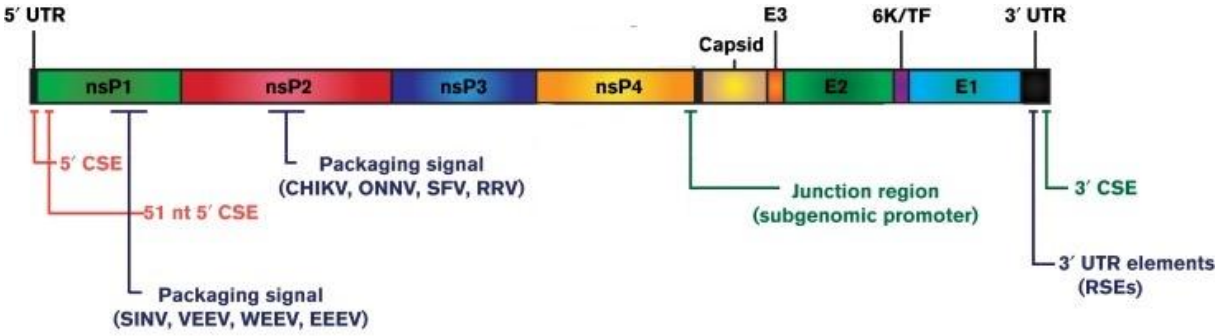

Figure 3. Genomic organisation of an alphavirus, highlighting coding regions and key regulatory RNA elements. The genome is flanked by 5’ untranslated region (5’-UTR) and 3’ untranslated region (3’-UTR). At the 5’ end, non-structural proteins (nsP1-nsP4) are encoded. Downstream of the nsP sequences, the structural polyprotein is encoded, including capsid protein, envelope proteins (E1-E3), and 6K/TF protein. A 5’ conserved sequence element (5’CSE) represents CSE1 and is located at the 5’ end. Within the nsP1 sequence, 51 nt 5’ CSE depicts CSE2. A packaging signal is present at the nsP1 or nsP2 sequences because its location varies among alphavirus species. The junction region, also referred to as the SG promoter, separates the two ORFs. At the 3’ end, repeat sequence elements (RSEs) and 3’ CSE, known as CSE4, are shown. (Rupp *et al.*, 2015), with modifications.

1.3.2 Coding regions

Non-structural proteins

The primary function of nsPs is replication and transcription of the genomic RNA. During alphaviral infection of the cell, mature nsPs assemble into the replicase complex (RC), which forms a spherule (Luo *et al.*, 2025). Spherules are bubble-like structures that house the RC and are the sites of viral RNA synthesis (Figure 4) (Leung *et al.*, 2011).

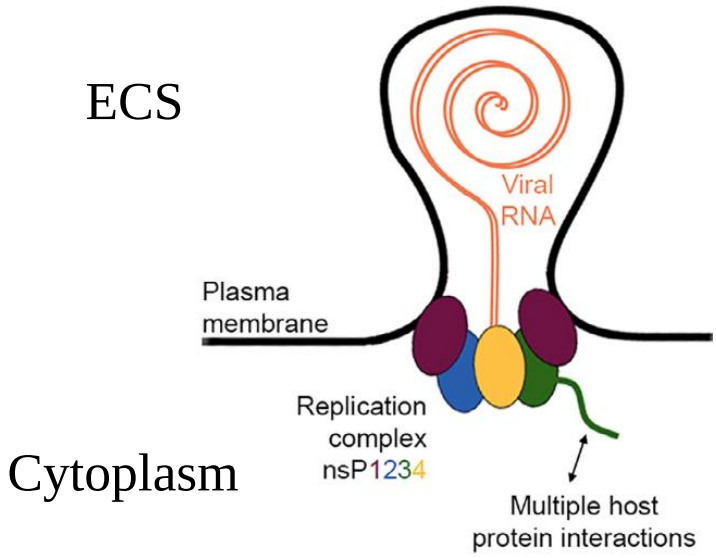

Figure 4. Schematic visualisation of the alphavirus spherule structure. The alphavirus replication complex forms a spherule at the plasma membrane. ECS is an abbreviation for the extracellular space. (Ahola *et al*., 2021), with modifications.

Within the RC, nsP1 assembles into a dodecameric ring that forms and stabilises the spherule neck (Ahola *et al.*, 2021). Through the strong affinity of nsP1 for the host cell's membrane, it interacts with negatively charged phospholipids in the host cell's plasma membrane, promoting the spherule formation (Rupp *et al.*, 2015). In addition, nsP1 functions as a viral RNA capping enzyme by generating the cap-0 structure on viral RNAs. This structure enables viral RNAs to evade host immune defences by mimicking host mRNA, facilitating efficient translation by host ribosomes, and protecting viral RNAs from degradation by host enzymes (Luo *et al.*, 2025).

After viral RNA translation, nsP2 is responsible for well-ordered cleavage of the ns-polyprotein into mature nsPs. During RNA replication, nsP2 performs multiple functions, including hydrolysing nucleoside triphosphates (NTPs) to generate energy used for unwinding double-stranded RNA replication intermediates. This activity enables the viral RNA polymerase (nsP4) to access the viral RNAs and synthesise new genomic and sub-genomic RNAs (SG RNAs) (Wang, Mahalingam *et al.*, 2025).

nsP3 is historically the least known among alphaviral nsPs (Ahola *et al.*, 2021). The nsP3 facilitates effective viral RNA replication by recruiting various host proteins to the replication site (Luo *et al.*, 2025).

nsP4 is the most conserved of the alphaviral nsPs (Ahola *et al.*, 2021). It is believed to serve as the primary interaction hub for other nsPs during formation of the RC (Tan *et al.*, 2022). However, its primary function is an RNA-dependent RNA polymerase (RdRp), responsible for

synthesising negative-strand RNA, genomic RNA, and SG RNA (Tan *et al.*, 2022). The RdRp sequence contains the highly conserved GDD (Glycine-Aspartic acid-Aspartic acid) motif characteristic of alphaviral polymerases (*Tomar et al.*, 2006). Mutation of this catalytic motif to GAA (Glycine-Alanine-Alanine) completely inactivates the enzymatic activity of nsP4 (Tomar *et al.*, 2006).

Structural proteins

Glycoproteins E1 and E2 form E1-E2 heterodimers that group into trimers, thereby forming spikes (Figure 5) (Rupp *et al.*, 2015). The trimetric spikes are bound to the alphavirus lipid envelope (Strauss & Strauss, 1994). Specifically, during alphaviral entry, E2 binds to receptors on the host cell surface (Mendes & Kuhn, 2018). During the alphaviral genome release, the cytoplasmic tail of E2 interacts directly with the nucleocapsid core, triggering the budding of new virions (Mendes & Kuhn, 2018). Moreover, E1 mediates the merging of the viral envelope with the endosomal membrane during the viral entry (Luo *et al.*, 2025). Finally, E3 is a signal sequence required for the proper translation of structural protein sequences (Mendes & Kuhn, 2018).

Capsid protein (CP) is a multifunctional structural protein of the alphavirus virion (Figure 6). CP can act as a protease that cleaves itself from the structural polyprotein (Mendes & Kuhn, 2018). Cleaved CP governs genome packaging by recognising the PS of viral RNA (Mendes & Kuhn, 2018). Structurally, CP assembles the icosahedral nucleocapsid core, which encapsulates and protects the viral genome (Luo *et al.*, 2025).

6K and Transframe (TF) proteins are membrane-associated components of alphavirus structural polyproteins (Figure 5) (MacLachlan & Dubovi, 2017). The 6K protein acts as a transmembrane ion channel (viroporin) necessary for the efficient budding and release of new virions (Bhardwaj *et al.*, 2026). TF is produced by a ribosomal frameshift during translation of the 6K coding sequence (Firth *et al.*, 2008). The TF protein is palmitoylated and plays an important role in virion maturation (Bhardwaj *et al.*, 2026). However, many of the 6K and TF functions remain unconfirmed or even unknown (Bhardwaj *et al.*, 2026).

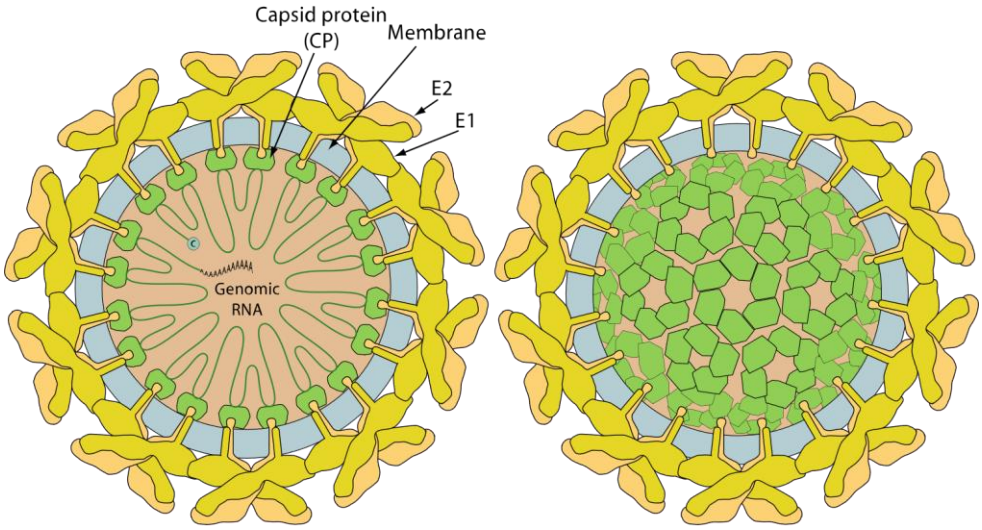

Figure 5. Schematic illustration of alphaviral virion in cross-sectional view. The left side depicts the association of the packaged viral genome and the capsid protein (CP). The right side represents the structural organisation of the icosahedral nucleocapsid. E1 (yellow) and E2 (orange) assemble into trimers of heterodimers embedded in the viral membrane bilayer (grey). E2 interacts with the capsid protein (CP, green), which assembles with genomic RNA (green line). This assembles the viral nucleocapsid. (ViralZone, n.d.).

1.4 INFECTION CYCLE OF ALPHAVIRUSES

Alphavirus infection begins when an infected insect vector bites the host. The alphavirus enters cells by attaching its trimeric spikes to cellular receptors, triggering clathrin-mediated endocytosis and membrane fusion that releases viral RNA into the cytoplasm (Figure 6A) (Luo *et al.*, 2025).

Viral RNA possesses a cap-0 and poly-A tail, which are essential for recognition by host ribosomes and subsequent translation of viral RNA into ns-polyproteins (Ahola *et al.*, 2021). While still embedded in the ns-polyprotein, nsP1 anchors the complex to the membrane to initiate spherule formation (Ahola *et al.*, 2021). nsP2 sequentially cleaves the polyprotein at three sites (Figure 6B) (Wang, Mahalingam *et al.*, 2025). Firstly, nsP2 cuts at the 3/4 site to release mature nsP4 and form the early RC composed of P123 and nsP4 (Figure 6B) (Lello *et al.*, 2021). The early RC recognises CSE4 at genomic RNA and initiates the synthesis of negative-strand RNA (Rupp *et al.*, 2015; Luo *et al.*, 2025). Secondly, nsP2 cleaves at the 1/2 and 2/3 sites, releasing mature nsP1, nsP2, and nsP3, which form the late RC (Figure 6B) (Wang, Mahalingam *et al.*, 2025; Lello *et al.*, 2021). The late RC is anchored at the narrow neck of the spherule (Figure 4) (Ahola *et al.*, 2021). which Late RC recognises CSE1, which functions as the promoter for positive-strand genomic RNA synthesis, and SG promoter, which promotes SG RNA synthesis (Rupp *et al.*, 2015; Frolov *et al.*, 2001). Using the negative-

stranded RNA as a template, nsP4 synthesises new positive-stranded RNAs and SG RNA (Lello *et al.*, 2021). The newly synthesised RNAs are capped and pumped out through the spherule's neck into the cytoplasm (Figure 6B) (Ahola *et al.*, 2021). The positive-stranded genomic RNAs are either used to further translation into ns-polyproteins or packaged into new virions (Strauss & Strauss, 1994).

In the cytoplasm, host ribosomes recognise SG RNA as a host mRNA and translate it into the structural polyprotein CP-E3-E2-6K/TF-E1 (Luo *et al.*, 2025). The CP cleaves itself and recruits the remaining polyprotein to the endoplasmic reticulum (ER), where it is processed into mature E1 and E2 glycoproteins that form fusion-competent trimeric spikes (Mendes & Kuhn, 2018). Meanwhile, cytoplasmic CP recognises the packaging signal (PS) on genomic RNA (not SG or cellular RNAs), driving CP multimerisation around one genomic RNA copy to form the nucleocapsid (NC) (Bhardwaj *et al.*, 2026). The NC interacts with the cytoplasmic tails of E2 at the plasma membrane, initiating budding: the membrane envelops the NC, forming the viral envelope (Figure 6) (Luo *et al.*, 2025). The mature virion is then released from the cell and is ready to infect new cells (Figure 6).

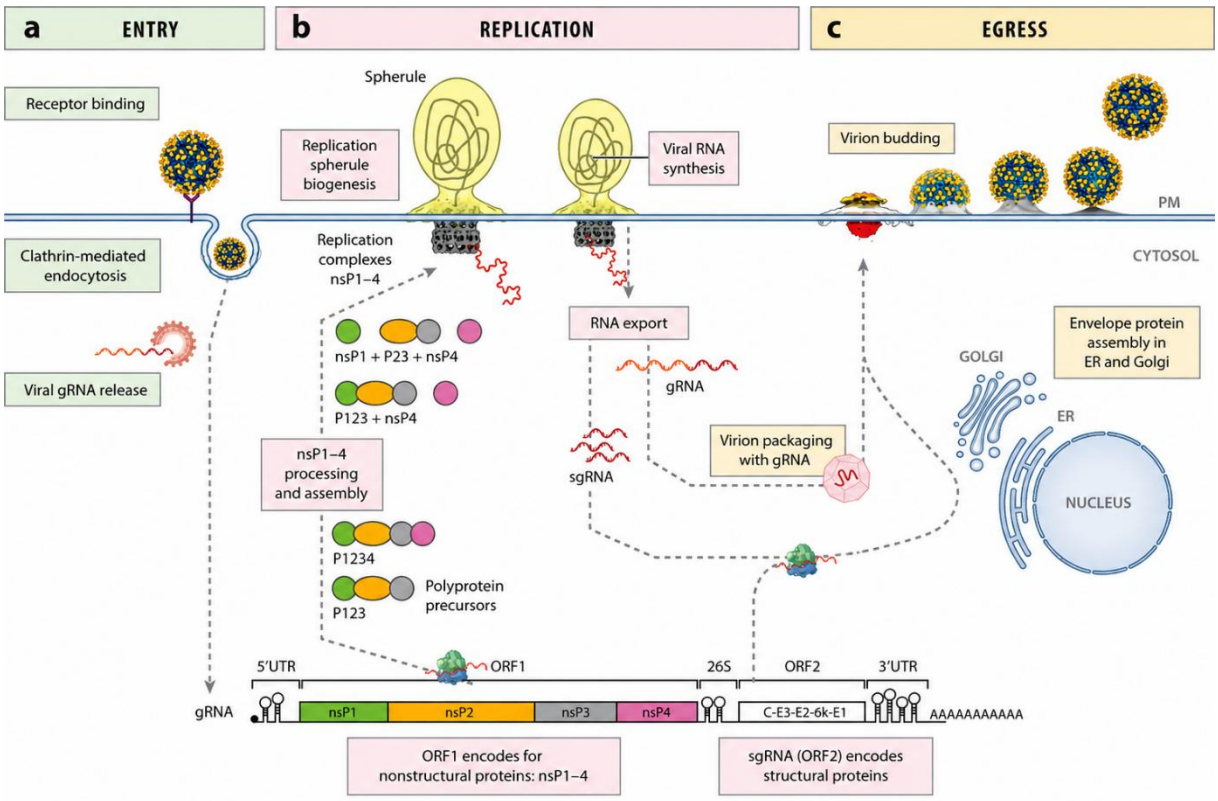

Figure 6. Schematic flow of the alphavirus infection cycle. (A) Alphavirus binds to a specific receptor on the host cell membrane. It triggers clathrin-mediated endocytosis. Viral and cell membranes fuse. The nucleocapsid disintegrates in the cytoplasm, releasing genomic RNA (gRNA). (B) gRNA is translated into the ns-polyprotein, which is spliced and assembled into the RC, promoting spherule biogenesis. The RC uses gRNA as a template to synthesise negative (-) strand RNA. (-) RNA is used to synthesise multiple genomic RNAs and sub-genomic (SG) RNA, which are released from the spherule. SG RNA is translated into structural proteins. (C) The capsid protein (C) self-cleaves and assembles into the nucleocapsid. The remaining structural proteins are processed in the endoplasmic reticulum (ER) and the Golgi complex. Mature envelope proteins are assembled into trimeric spikes. The nucleocapsid binds to trimeric spikes on the cell membrane, triggering budding. The cell membrane is wrapped around the nucleocapsid. Mature virion is released (Luo *et al.*, 2025).

1.5 IMPORTANCE OF ALPHAVIRUS RESEARCH

The increase in the incidence of human diseases caused by alphaviruses is a growing global health concern due to their potential for large outbreaks. Their spread is partly driven by climate change. Transmission risks are increased by rising temperatures, which allow mosquito vectors to expand into new areas. In 2025, there were 445 271 CHIKV infections reported along with 155 deaths in 40 countries (World Health Organization, 2025). Additionally, Finland recorded 566 SINV infections in 2021, meaning that alphaviral infection outbreaks are not restricted to tropical regions (Suvanto *et al.*, 2022).

Alphavirus infections have been a major issue for many decades since there was no vaccine or antiviral medication against them (Bassetto & Brancale, 2021). The treatment was mainly symptomatic. The situation was improved with the approval of two CHIKV vaccines in 2023 (European Medicines Agency, 2025) and 2025 (Bavarian Nordic, 2025). This breakthrough marks the first time prevention of an alphavirus infection has become possible.

In contrast, insect-specific alphaviruses such as EILV and TALV have not been associated with infections in humans or other vertebrates (Hermanns *et al.*, 2020). Nevertheless, studying these viruses can provide valuable insights into the fundamental mechanisms of alphavirus replication and evolution. Therefore, it will enhance our understanding of the biology, evolution, and pathogenic potential of medically important alphaviruses, thereby improving antiviral research.

1.6 TALV

TALV was discovered in 2004 during mosquito surveillance in Taï National Park, the Ivory Coast. The virus was detected in *Culex decens* mosquitoes. Only one positive pool, containing 19 *Culex decens* mosquitoes, was identified among 8,860 female mosquitoes tested across three tropical regions (Mexico, Uganda, and the Ivory Coast). Consequently, these findings suggest that TALV is an insect-specific alphavirus and is presumably extremely rare (Hermanns *et al.*, 2017).

The scientists who discovered TALV were unable to propagate the virus in both *Aedes albopictus* (C6/36) and African green monkey kidney (Vero) cell lines (Hermanns *et al.*, 2017). Based on the experimental conditions tested, researchers suggested that TALV has a narrow host range (Hermanns *et al.*, 2017). To date, no stable cultured isolate of TALV has been established despite various trials (Bell *et al.*, 2024). On the other hand, insect-specific alphaviruses such as EILV can replicate efficiently in mosquito cells *in vitro*.

To identify the origin of the TALV, researchers used RNA extracted directly from the mosquito pool homogenate to reconstruct the TALV genome. Phylogenetic analyses, based on the ORF of the structural protein, identified TALV as a sister lineage to EILV (Figure 1). These two viruses share approximately 67% nucleotide identity (Hermanns *et al.*, 2017).

Moreover, phylogenetic analysis indicated that TALV occupies a basal position relative to the WEEV complex (Figure 1). This suggests that TALV separated from the lineage before the

WEEV complex diversified into its current members. This conclusion was derived through comparative genomic analyses of TALV across alphavirus species. In addition, the results revealed high levels of identity between the CSEs and the RSEs in the TALV genome and in the genomes of the members of the SFV and WEEV complexes (Table 1). For example, TALV RSEs show sequence similarity to RRV-type RSEs, which are common among SFV complex (Hermanns *et al.*, 2017).

Table 1. Conservation levels of TALV genomic elements. The table highlights the conservation levels of TALV genome elements relative to those of different alphaviruses (Hermanns *et al.*, 2017).

Genomic element	**Conservation level**
CSE4	Universally conserved
CSE2	77-92% identity to EILV, SINV, WEEV, RRV, CHIKV
RNA-dependent RNA polymerase (RdRp) motifs	Resembles EILV
RSEs	Like EILV-type and RRV-type
Sub-genomic (SG) promoter	71% identity to EILV

Despite these studies, TALV remain one of the least well-characterised alphaviruses in the *Togaviridae* family.

1.7 TRANS-REPLICASE ASSAY

Introduction to the trans-replicase assay

Work with alphaviruses may be limited by concerns about biosafety, as multiple species within the genus are human pathogens (Strauss & Strauss, 1994). To overcome this, trans-replicase assays have been developed, allowing for experimental manipulation at lower biosafety levels (Wang, Naumenko *et al.*, 2025).

In a standard infection, viral RNA acts as a messenger for protein synthesis and a template for viral replication (Ahola *et al.*, 2021). In the standard trans-replicase assay, these functions can be separated into two plasmids: a replicase and an RNA template (minigenome) (Utt *et al.*, 2016). The replicase plasmid encodes the viral ns-polyprotein, which drives replication (Utt *et al.*, 2019). The minigenome plasmid lacks ORFs for ns- and structural genes (Utt *et al.*, 2019). Instead, the minigenome contains *cis*-acting RNA elements and reporter genes (Lello et al., 2021). *Firefly* (FLuc) and *Gaussia* (GLuc) luciferases are frequently used as reporters of expression for ns-genes and structural genes, respectively (Lello *et al.*, 2021). Hence, FLuc and GLuc enable quantification of viral replication and analysis of replicase-minigenome interactions and *cis*-acting elements.

Promoter systems used in the trans-replicase assay

The expression of these plasmids depends on the careful selection of the promoter systems. Promoters differ in which host RNA polymerases recognise them, and this specificity is essential for controlling when RNA is produced from each plasmid. Inappropriate promoter selection can yield artificial results by leading to premature or excessive reporter expression before the viral replicase is mature.

Typically, replicase plasmids use promoters recognised by host RNA polymerase II (RNA PolII), which produce capped transcripts, ensuring effective recognition by host ribosomes (Lello *et al.*, 2021). These include cytomegalovirus (CMV) promoter in mammalian cells and Ubi (*Aedes aegypti* polyubiquitin) promoter in insect cells (Bartholomeeusen *et al.*, 2018; Utt *et al.*, 2016). On the other hand, minigenome plasmids typically encode the promoters recognised by the host RNA polymerase I (RNA PolI), which generate noncapped transcripts that are poorly translated by host ribosomes (Lello *et al.*, 2021). These include HSPolI (*Homo sapiens* RNA polymerase I promoter) in mammalian cells and AlbPolI (*Aedes albopictus* RNA polymerase I promoter) in insect cells (Utt *et al.*, 2019). Therefore, HSPolI or AlbPolI ensure that reporter expression is low before the viral replicase is present. This design successfully prevents the artificial background signal. However, depending on the experimental conditions and the goal, other promoter systems can also be applied in the plasmids. The structure of the minigenome and replicase plasmid is described in detail in the Materials and Methods.

Standard trans-replicase assay During a standard trans-replicase assay (Figure 7), cells are co-transfected with both replicase and minigenome plasmids. First, promoters on the replicase plasmids are recognised by RNA Pol II, producing capped transcripts. Simultaneously, host

RNA PolI recognises the promoters of minigenome plasmids and transcribes them, producing noncapped transcripts. Host ribosomes efficiently translate capped transcripts, producing ns-polyproteins. These polyproteins are processed and assembled into the early RC. It detects the minigenome RNA and synthesises a negative-strand intermediate (Wang, Naumenko *et al.*, 2025). Once the early RC is fully processed, it matures into late RC, which produces both full-length minigenomic RNA and shorter SG RNA from the negative-strand intermediate (Utt *et al.*, 2019). The SG RNA is translated into GLuc, reflecting transcriptional activity (Utt *et al.*, 2016). At the same time, FLuc indicates replication of full-length RNA (Utt *et al.*, 2016). Luciferase activity is measured using the dual-luciferase reporter assay system, which provides quantitative measurements of viral replication (FLuc) and transcription (GLuc).

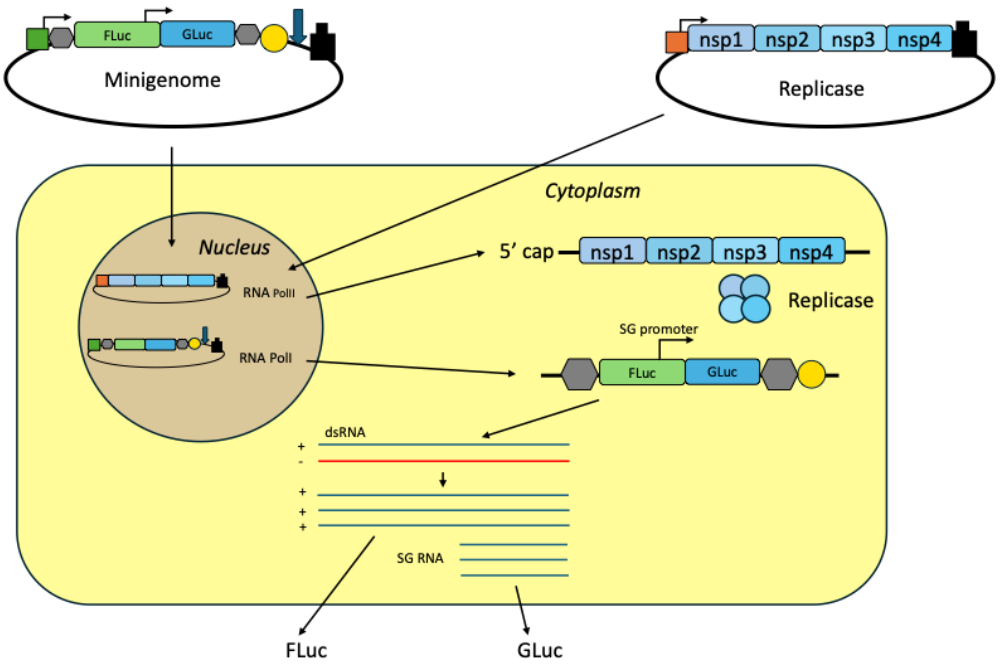

Figure 7. Trans-replicase assay scheme. After entering the cell, both minigenome and replicase plasmids are transported into the nucleus. There, the CMV or Ubi promoter (orange square) on the replicase plasmid is recognised by RNA polymerase II (RNA Pol II), leading to transcription of the replicase plasmid. Simultaneously, the HSPolI or AlbPolI promoter (dark green square) on the minigenome plasmid is recognised by RNA polymerase I (RNA PolI), resulting in the transcription of the minigenome. In the cytoplasm, the transcripts are translated. The replicase complex recognises the minigenome RNA to generate negative-strand (-) RNA intermediates. These (-) RNA templates are subsequently used to produce positive-strand (+) RNA, leading to FLuc expression and activity. Replicase recognises the sub-genomic (SG) promoter and transcribes SG RNA, leading to GLuc expression and activity. (Lello, PhD thesis), with modifications.

Advantages of the standard trans-replicase assay

The standard trans-replicase assay provides controlled, safe experimental conditions for studying viral replication. However, it also offers more experimental advantages. Firstly, the

assay allows mixing and matching replicases and minigenomes from distinct alphaviruses to study their functional compatibility and evolutionary relationships (Utt *et al.*, 2019). Secondly, it can be used to analyse mutations that would be lethal in infectious viruses (Wang, Naumenko *et al.*, 2025).

Two-component trans-replicase assay

While the standard trans-replicase system effectively models natural viral replication, researchers have developed variations of this trans-replicase approach to gain greater experimental control and insight into specific viral functions. To specify, the standard trans-replicase system, often referred to as a one-component replicase, expresses all four non-structural proteins (nsP1-4) from a single plasmid as a P1234 polyprotein (Utt *et al.*, 2016). In contrast, the split trans-replicase assay separates these components by expressing them from multiple plasmids (Utt *et al.*, 2019). For example, in a two-component system, the replicase is divided into P123 and nsP4 (Utt *et al.*, 2019). The mechanism of this assay is illustrated in Figure 8. Overall, this approach improves experimental specificity by allowing individual replicase components to be studied separately. For example, replicase modules from different alphaviruses (e.g., P123 from TALV and nsP4 from CHIKV) can be combined to assess functional compatibility and identify which proteins recognise the minigenome.

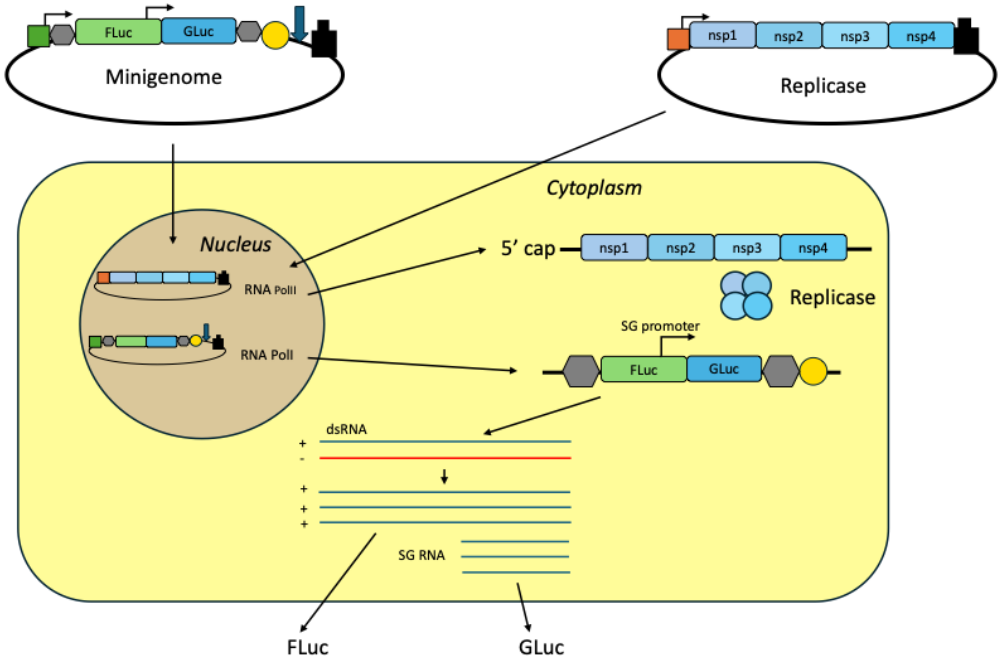

Figure 8. Split trans-replicase assay scheme. After entering the cell, both the minigenome and the nsP123 replicase and nsP4-GDD plasmids are transported into the nucleus. There, the CMV or Ubi promoter (orange square) on the GAA and nsP4 plasmids is recognised by RNA polymerase II (RNA Pol II), leading to transcription of these plasmids. Simultaneously, the HSPolI or AlbPolI promoter (dark green square) on the minigenome plasmid is recognised by RNA polymerase I (RNA PolI), resulting in the transcription of the minigenome. In the cytoplasm, the transcripts are translated. The GAA replicase complex pairs with nsP4 to assemble a fully functional replicase complex. This complex recognises the minigenome RNA to generate negative-strand (-) RNA intermediates. These (-) RNA templates are subsequently used to produce positive-strand (+) RNA, leading to FLuc expression and activity. Replicase recognises the sub-genomic (SG) promoter and transcribes SG RNA, leading to GLuc expression and activity.

In conclusion, the trans-replicase assay is an effective method for studying emerging viruses such as TALV. The usage of this method provides insights into viral replication mechanisms, cross-species compatibility, and host-related factors (Lello *et al.*, 2021).

II Experimental part

2.1 AIMS OF THE THESIS

TALV represents a poorly characterised member of the *Alphavirus* genus. The experimental studies of TALV have been severely constrained by the absence of a stable cultured isolate, with only one complete genome sequence reported to date.

Trans-replicase assays provide a powerful approach for studying viral RNA replication under controlled conditions without handling infectious virus.

This thesis aimed to:

- Develop the TALV trans-replicase system in two cell lines, U2OS mammalian cells and C6/36 insect cells.

- To cross-compare TALV with other alphaviruses to assess:
    - temperature sensitivity,
    - compatibility,
    - specificity.

2.2 MATERIALS AND METHODS

2.2.1 Molecular cloning of TALV minigenomes and replicases for mammalian and insect models

The design of minigenome and replicase plasmids

In previous work conducted by our research group, the cloning strategy was designed, and synthetic DNA fragments were ordered by A. Merits. These fragments were subsequently cloned to generate plasmids suitable for both mammalian and insect cell models. Minigenome and replicase plasmids for both mammalian and insect cells were successfully constructed using restriction enzyme-based cloning, as illustrated in Figures 10 and 11.

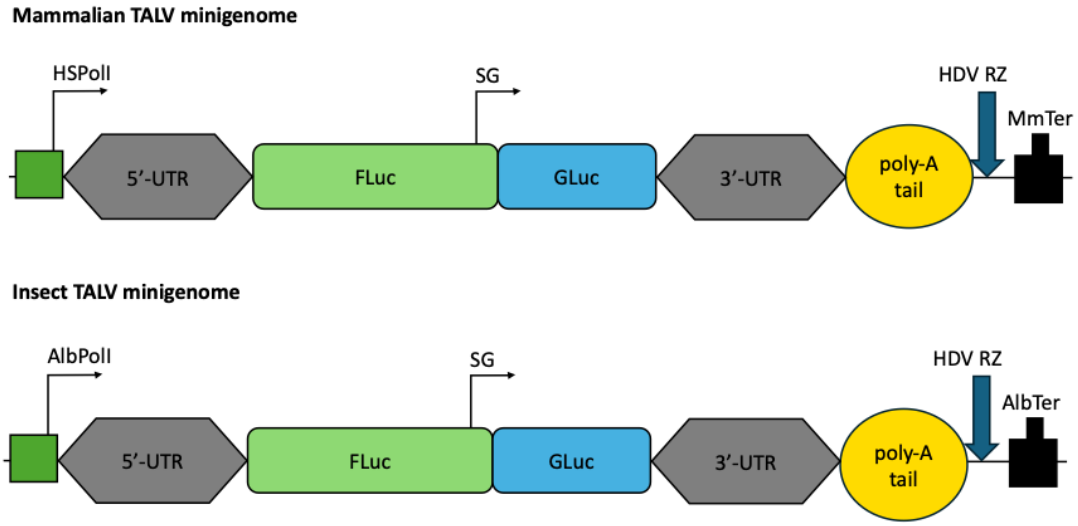

Figure 10. Schematics of minigenome plasmids for mammalian and insect models. At the 5' and 3' ends of each minigenome, host-specific promoters and terminators are located for the recognition by host RNA PolI. The mammalian model comprises the HSPolI promoter and the mammalian terminator (MmTer). The insect model comprises the AlbPolI promoter and the *Albopictus* terminator (AlbTer). Other components of the plasmid include 5'-UTR and 3'UTR, *Firefly* luciferase (FLuc), sub-genomic promoter (SG), *Gaussia* luciferase (GLuc) and poly-A tail. A hepatitis delta virus antisense strand ribozyme (HDV RZ) is introduced to ensure the minigenome is precisely cleaved, eliminating the terminator sequence. Size not to scale.

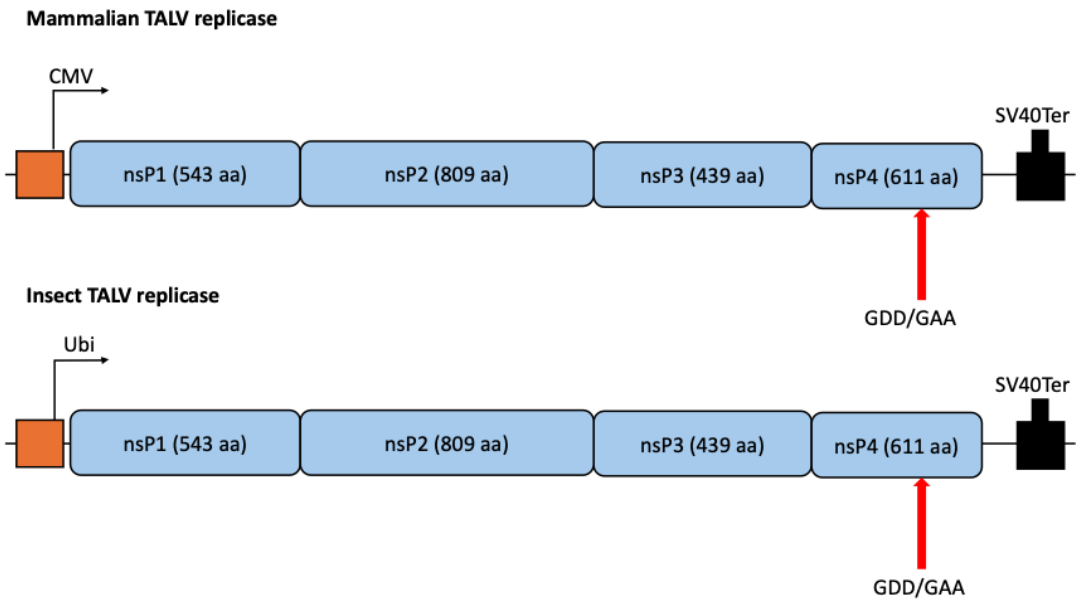

Figure 11. Schematics of replicase plasmids for mammalian and insect models. At the 5' end of each replicase, host-specific promoters and the Simian virus 40 terminator (SV40Ter) are located for recognition by host RNA PolII. The mammalian model comprises the CMV promoter, and the insect model – the Ubi promoter. Also, the plasmid includes the nsP1-nsP4 sequences. The red arrow indicates a GDD motif, corresponding to a wild-type (wt) replicase plasmid, or to point mutations (GAA) in the nsP4 sequence, representing a polymerase-inactive replicase plasmid. Size not to scale.

Molecular cloning for the TALV minigenome and replicase plasmids

The designed plasmids were constructed using a conventional cloning strategy and restriction enzymes (Thermo Fisher Scientific). The enzymes were used according to the manufacturer's instructions. To prevent self-ligation, linearised vector fragments were dephosphorylated using FastAP Alkaline Phosphatase (Thermo Fisher Scientific). Meanwhile, the agarose gel-extracted insert fragments were purified with the Zymoclean Gel DNA Recovery Kit (Zymo Research), following the manufacturer's instructions.

DNA ligation was performed to ligate the purified insert fragments into the prepared vector fragments. Ligation reactions were carried out using T4 DNA ligase (Thermo Fisher Scientific). Following ligation, the resulting reaction mixtures were used to transform competent *E. coli* cells via heat shock.

Transformation and plasmid amplification

E. coli XL10 competent cells were used for plasmid propagation. Aliquots of competent cells were thawed on ice and mixed with the ligation reaction mixture. The bacterial cells were incubated on ice for 30 minutes, followed by heat shock at 42°C for 120 seconds. Cells were immediately returned to ice for 3 minutes. Subsequently, SOC media was prepared by

combining SOB (Super Optimal Broth, Thermo Fisher Scientific) with 0.4% glucose. SOC media was added, and the cells were incubated at 37°C for 45 minutes with shaking at 200 rpm. Furthermore, transformed bacterial cells were plated on lysogeny broth agar (LB) plates supplemented with appropriate antibiotics and incubated overnight at 37°C.

Colonies were selected, and plasmids were isolated using the FavorPrep™ Plasmid Extraction Mini Kit (Favorgen), following the manufacturer's protocol. The resulting plasmids were screened via restriction enzyme digestion to confirm the presence and orientation of the insert. Confirmed clones were then cultured overnight in LB medium to amplify the plasmid DNA for subsequent purification. Large-scale plasmid isolation was performed using the NucleoSnap Plasmid Midi kit (Macherey-Nagel) according to the manufacturer's protocol.

Verification and validation of recombinant constructs

The final constructs included the minigenome and its respective replicases for mammalian and insect models (Table 2). The plasmids were verified via Whole Plasmid Sequencing (Plasmidsaurus, Inc.). The resulting sequences were validated by aligning them against reference sequences in the NCBI GenBank database using Benchling (Benchling, Inc.) to ensure sequence integrity.

Table 2. Final sequences of mammalian and insect models. The table depicts the construct type (e.g., minigenome or replicase), the plasmid name, and the sequence length in base pairs (bp). The construct type includes a minigenome, a polymerase-active replicase plasmid (wt), and a polymerase-inactive replicase plasmid (GAA). The plasmid name abbreviates the promoter system, the TALV affiliation, and the construct type. TempFG indicates the template (a minigenome encoding *Firefly* and *Gaussia* luciferases as reporters), and Repl specifies the replicase.

Model	Construct type	Plasmid name	Size (bp)
Mammalian	Minigenome	HSPolI-TALV-TempFG	6053
	Replicase wt	CMV-TALV-Repl-wt	14 980
	Replicase GAA	CMV-TALV-Repl-GAA	14 980
Insect	Minigenome	AlbPolI-TALV-TempFG	5898
	Replicase wt	Ubi-TALV-Repl-wt	13 202
	Replicase GAA	Ubi-TALV-Repl-GAA	13 202

For the trans-replicase assays, CHIKV, ONNV, RRV, SFV, MAYV, BFV, SINV, VEEV, EEEV, WEEV, and EILV constructs were used and generated in previous work conducted by our research group.

2.2.2 Cell culture experiments

Cultivation conditions and media

Human osteosarcoma cells (U2OS) were used for the transfection of mammalian constructs. The cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM, PAN biotech) supplemented with 10% fetal bovine serum (FBS, PAN biotech) and were incubated at 37°C or 28°C with 5% CO_2 supply.

Transfection of insect constructs was performed in the *Aedes albopictus* (C6/36) cells. C6/36 cells were maintained in Leibovitz's L-15 medium (PAN biotech) supplemented with FBS and 10% tryptose phosphate broth (TPB, Thermo Fisher Scientific) and were incubated at 28°C without a CO_2 supply.

Lipofectamine LTX-mediated transfection

Prior to the transfection, U2OS or C6/36 cells were seeded into 48-well plates and incubated until 70-80% confluency. The transfection complex was prepared through a sequential mixing procedure, according to the manufacturer's protocol. Plasmid was separately prepared in Opti-MEM® medium and complexed with PLUS™ reagent. Separately, Lipofectamine® LTX reagent (Thermo Fisher Scientific) was diluted in Opti-MEM™ medium (Thermo Fisher Scientific). These two solutions were combined to form the DNA-lipid complex. Subsequently, the cell line-specific culture medium was added to the complexes. This transfection solution was then dispensed into the 48-well plates seeded with cells. Transfected cells were incubated under conditions appropriate to each cell line. U2OS cells were maintained at 37°C and 28°C for 24 hours post-transfection. C6/36 cells were maintained at 28°C for 48 hours post-transfection.

2.2.3 Dual-luciferase reporter assay system

Cell lysis

After incubation, the growth medium was removed, and the cells were lysed *in situ* using Passive Lysis Buffer (PLB, Promega).

Reagent preparation

Luciferase Assay Reagent II (LAR II) and Stop & Glo® Reagent were prepared before use. The latter was prepared by diluting the 50x Stop & Glo® Substrate in the provided assay buffer.

Luminescence measurement

All assays were performed on a GloMax® 20/20 Single Tube Luminometer. To determine Firefly luciferase (FLuc) activity, 20 µL of LAR II was added to 4 µL of cell lysate. Following the initial measurement, 20 µl of Stop & Glo® Reagent was added to the same tube to quench the FLuc signal and simultaneously measure the activity of the second reporter, *Gaussia* luciferase (GLuc).

2.2.4 Trans-replicase assay analysis

FLuc and GLuc signals were measured in relative light units (RLUs). These RLUs obtained from the dual-luciferase assay were inserted into the Excel file. These raw data were used to compare the replication and transcription activity of the minigenome and replicase. To quantify replicase activity, fold changes were calculated by normalising the RLU obtained from samples expressing wt replicase to the corresponding RLU from samples expressing GAA replicase (Formula 1). The column graphs were constructed from a comparative analysis of each assay.

Formula 1. The equation for the calculation of fold change in replication/transcription activity of the wt replicase relative to the GAA replicase control.

$$fold\ change = \frac{RLU_{wt}}{RLU_{GAA}}$$

2.3 RESULTS AND DISCUSSION

2.3.1 Mammalian model

Primary testing of TALV trans-replication assay in U2OS cells

The developed mammalian TALV minigenome and replicase plasmids were tested in U2OS cells at 37°C and 28°C (Figure 12A and 12B, respectively) to determine whether TALV replication is temperature-sensitive. EILV minigenome and replicase plasmids were used as a positive control due to the close evolutionary relationship between EILV and TALV and the well-established trans-replicase system for this alphavirus in the research group.

As shown in Figure 12, the replication levels for EILV and TALV were higher at 28°C than at 37°C. These results suggest that, similarly to EILV, TALV replication is temperature-sensitive (Nasar *et al.*, 2012). It may indicate that TALV replication machinery operates more efficiently at temperatures close to those of its mosquito hosts.

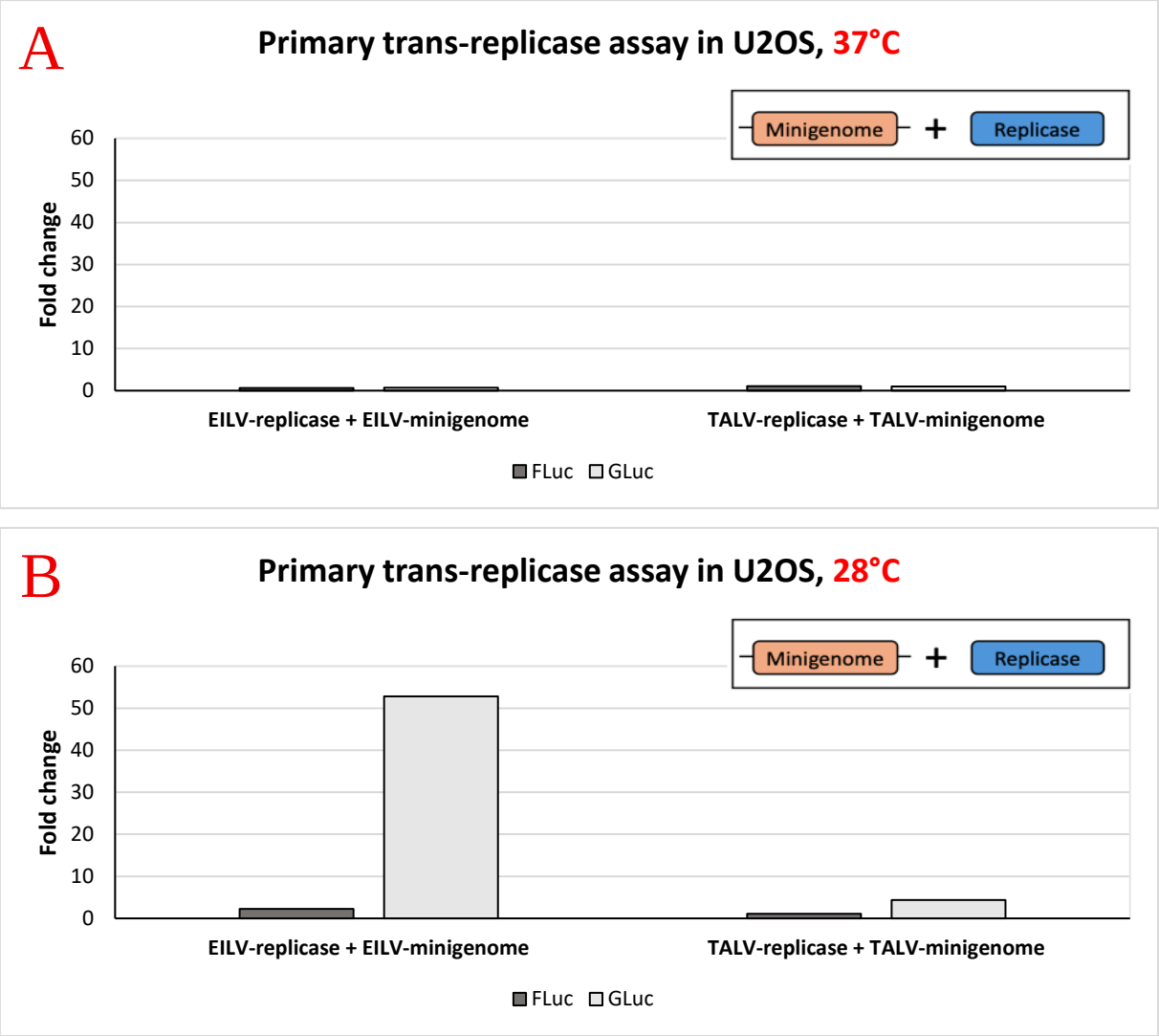

Figure 12. The primary data from the trans-replicase assay in U2OS cells at 37°C (A) and 28°C (B). Column graphs show the replication and transcription efficiency of EILV and TALV systems, measured by FLuc and Gluc luciferase reporter activity, respectively. The x-axis indicates the different minigenomes and replicase combinations. Fold change (y-axis) represents the ratio of RLU in cells transfected with an active viral replicase (wt) versus the control cells transfected with a polymerase-inactive (GAA) variant. The EILV is presented as a positive control.

Building on these findings, the TALV minigenome compatibility assay was conducted to assess the functionality of the mammalian TALV trans-replicase. Additionally, it may evaluate the ability of various alphavirus replicases to replicate the TALV minigenome.

Testing of TALV minigenome and different alphavirus replicases in the U2OS cells

This assay tested the compatibility of the TALV minigenome with different alphavirus replicases at 28°C and 37°C to evaluate phylogenetic relationships between TALV and other alphaviruses.

Figure 13 reveals that EILV and TALV reporter activity at 28°C was higher than at 37°C, confirming the trend observed in the primary testing. EILV replicase efficiently replicated the TALV minigenome at both temperatures, providing additional evidence for their close phylogenetic relationship. Beyond this, the CHIKV, RRV and SINV replicases were capable of driving TALV minigenome replication. These findings demonstrate that the TALV minigenome harbours *cis*-acting elements recognised by the members of SFV (CHIKV, RRV) and WEEV complexes. The SINV-TALV compatibility may support the conclusion that TALV occupies a basal position relative to the WEEV complex, suggesting a common evolutionary origin.

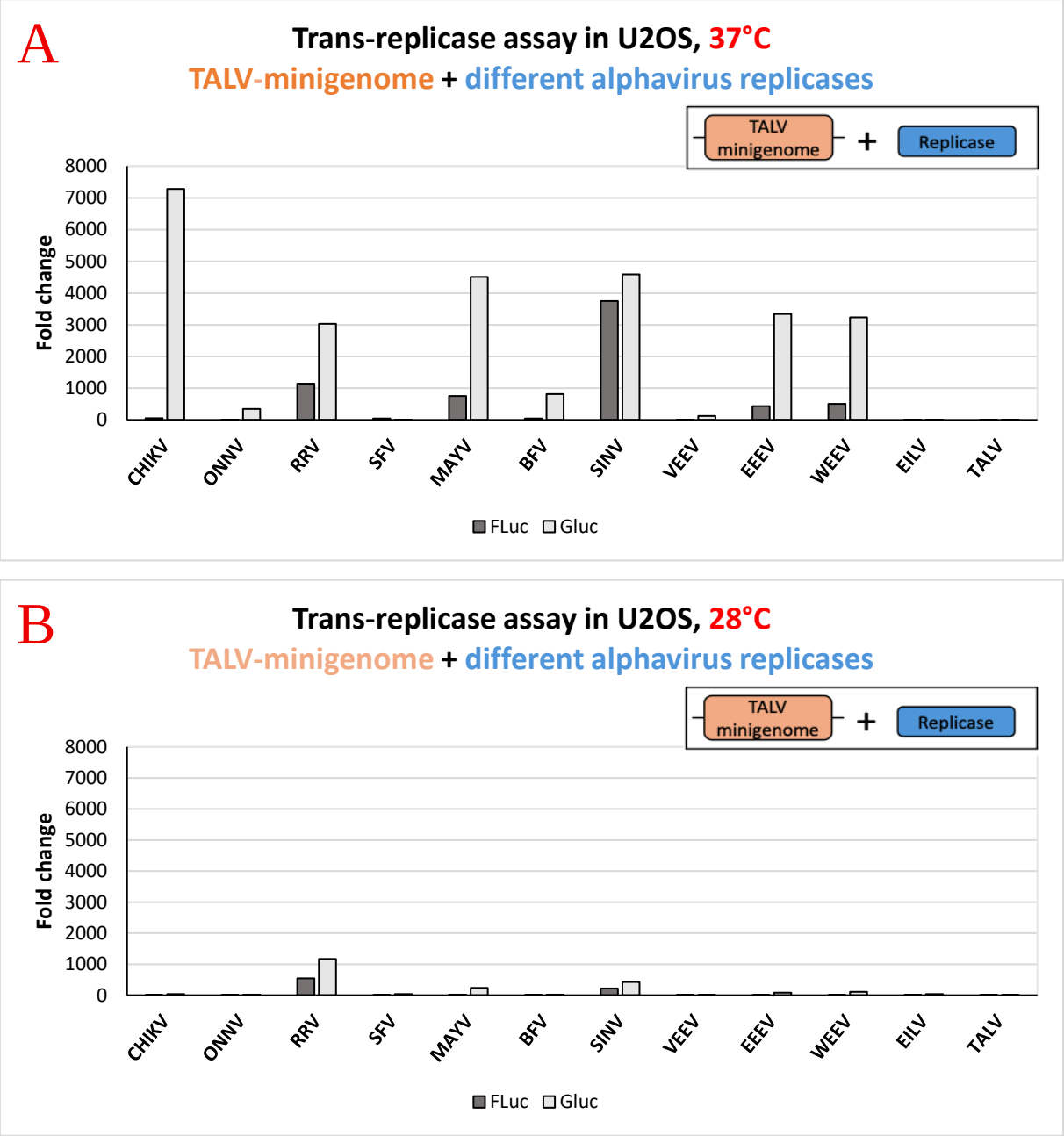

Figure 13. The data from the trans-replicase assay showing the compatibility of the TALV minigenome in U2OS cells at 37°C (A) and 28°C (B). Column graphs show the efficiency of alphaviral replicases in replicating and transcribing the TALV minigenome, measured by FLuc and Gluc luciferase reporter activity. The x-axis indicates the different minigenomes and replicase combinations. Fold change (y-axis) represents the ratio of RLU in cells transfected with an active viral replicase (wt) versus the control cells transfected with a polymerase-inactive (GAA) variant.

Testing of TALV replicases and different alphavirus minigenomes in the U2OS cells

The final step in the mammalian model testing was to conduct a reverse assay to investigate the compatibility of the TALV replicases with different alphavirus minigenomes. It may provide further insight into the phylogenetic relationships between TALV and other alphaviruses.

In contrast to previous compatibility in Figure 13, TALV replicase demonstrated extreme specificity for its respective and EILV minigenome (Figure 14). This suggests that TALV replicase exhibits specificity for *cis*-acting elements within the TALV minigenome or for unidentified requirements.

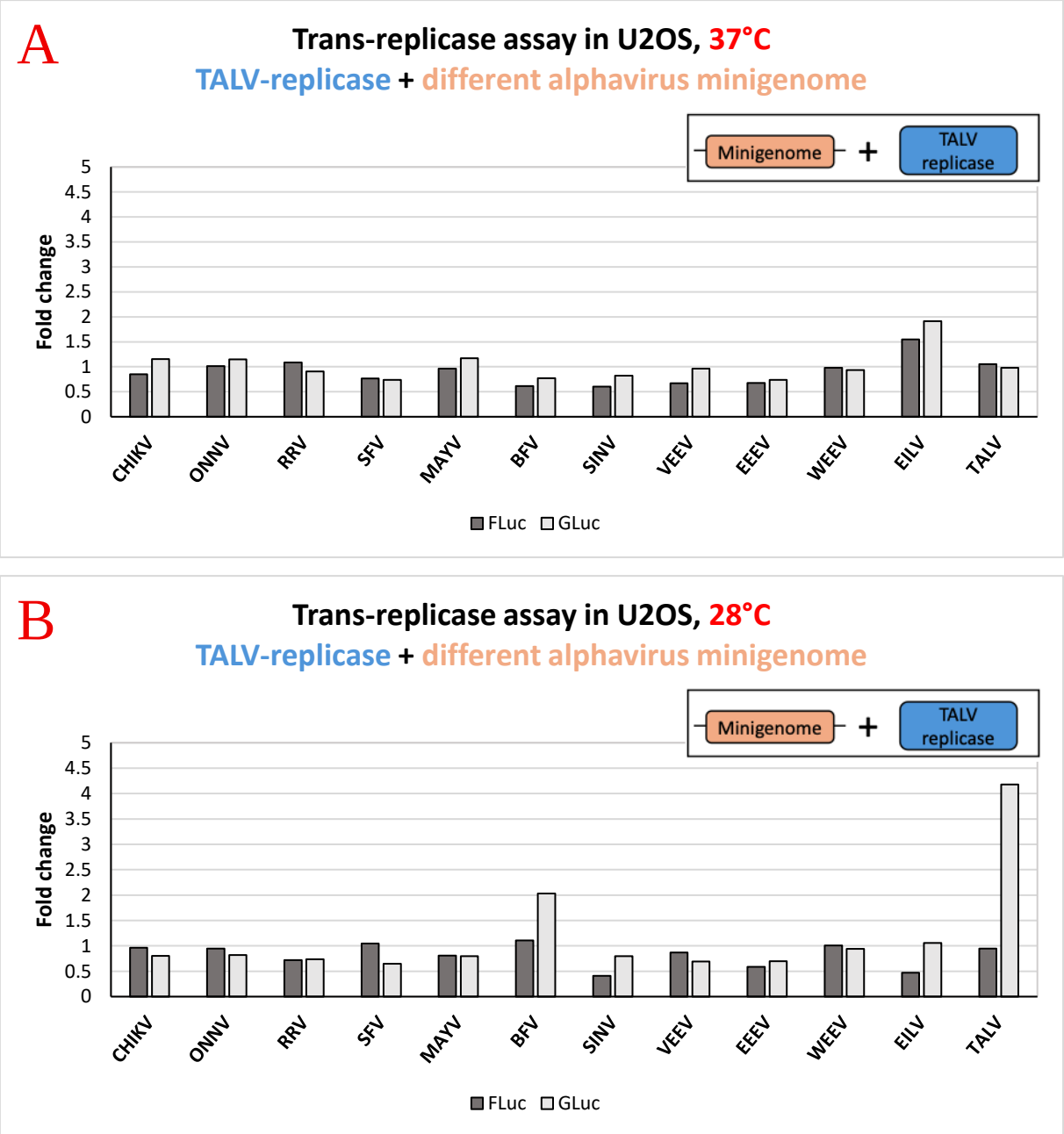

Figure 14. The data from the trans-replicase assay showing the compatibility of TALV replicase in U2OS cells at 37°C (A) and 28°C (B). Column graphs show the efficiency of TALV replicases in replicating and transcribing alphaviral minigenomes, measured by FLuc and Gluc luciferase reporter activity. The x-axis indicates the different minigenomes and replicase combinations. Fold change (y-axis) represents the ratio of RLU in cells transfected with an active viral replicase (wt) versus the control cells transfected with a polymerase-inactive (GAA) variant.

Taken together, these experiments indicate that the mammalian TALV trans-replicase assay is functional but displays temperature and replicase specificity. In addition, these results may indicate that TALV is relatively phylogenetically closest to EILV, close to the SFV complex (CHIKV, RRV), and basal to the WEEV complex.

Comparison across temperatures suggests that while 37°C served as a useful reference for assessing experimental conditions, 28°C is a more permissive temperature for TALV trans-replicase assays. However, genomic replication and sub-genomic transcription levels in U2OS cells remained reduced at 28°C, suggesting that an extended incubation period, such as 48 hours, may be necessary to improve the results.

Despite this, TALV replication efficiency by its respective minigenome and replicase plasmids was variable across experiments, indicating that the current mammalian model requires further optimisation. Future studies in additional mammalian cell lines, such as HEK293T or BHK-21, may help expand the understanding of TALV replication in mammalian models.

2.3.2 Insect model

Primary testing of TALV minigenome and replicases in the C6/36 cells

The developed insect TALV minigenome and replicase plasmids were transfected into C6/36 cells maintained at 28°C to assess the functionality and temperature sensitivity of the TALV replication machinery.

As shown in Figure 15, both EILV and TALV replicases demonstrated strong replication of their respective minigenomes, suggesting that 28°C may represent the optimal thermal conditions for the genomic replication and sub-genomic transcription of these viruses.

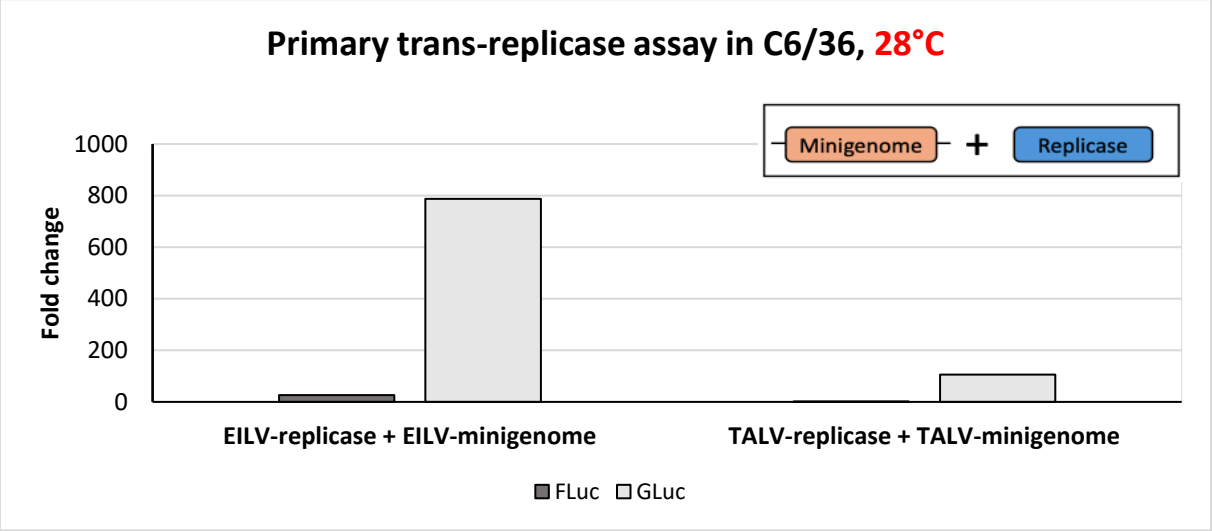

Figure 15. The primary data from the trans-replicase assay in C6/36 cells at 28°C. Column graphs show the replication and transcription efficiency of EILV and TALV systems, measured by FLuc and Gluc luciferase reporter activity. The x-axis indicates the different minigenomes and replicase combinations. Fold change (y-axis) represents the ratio of RLU in cells transfected with an active viral replicase (wt) versus the control cells transfected with a polymerase-inactive (GAA) variant. The EILV is presented as a positive control.

Building on these findings, further compatibility assays were conducted to further test the TALV trans-replicase assay as a functional platform for investigating alphavirus replication. It may evaluate the phylogenetic proximities of TALV to various alphaviruses.

Testing of TALV minigenome and different alphavirus replicases in the C6/36 cells

To determine whether TALV minigenome *cis*-acting elements are recognised by alphaviral replicases, TALV minigenome compatibility was tested against the various alphaviral replicases.

Figure 16 depicts that replicases of SFV complex (CHIKV, RRV) yielded measurable reporter activity, indicating conservation of *cis*-acting elements between SFV complex and TALV. EILV replicase continued to show strong cross-recognition, as expected from the close phylogenetic relationship of EILV and TALV. Notably, SINV replicase efficiently drove TALV minigenome replication. This cross-compatibility data may support the conclusion that TALV occupies a basal position relative to the WEEV complex.

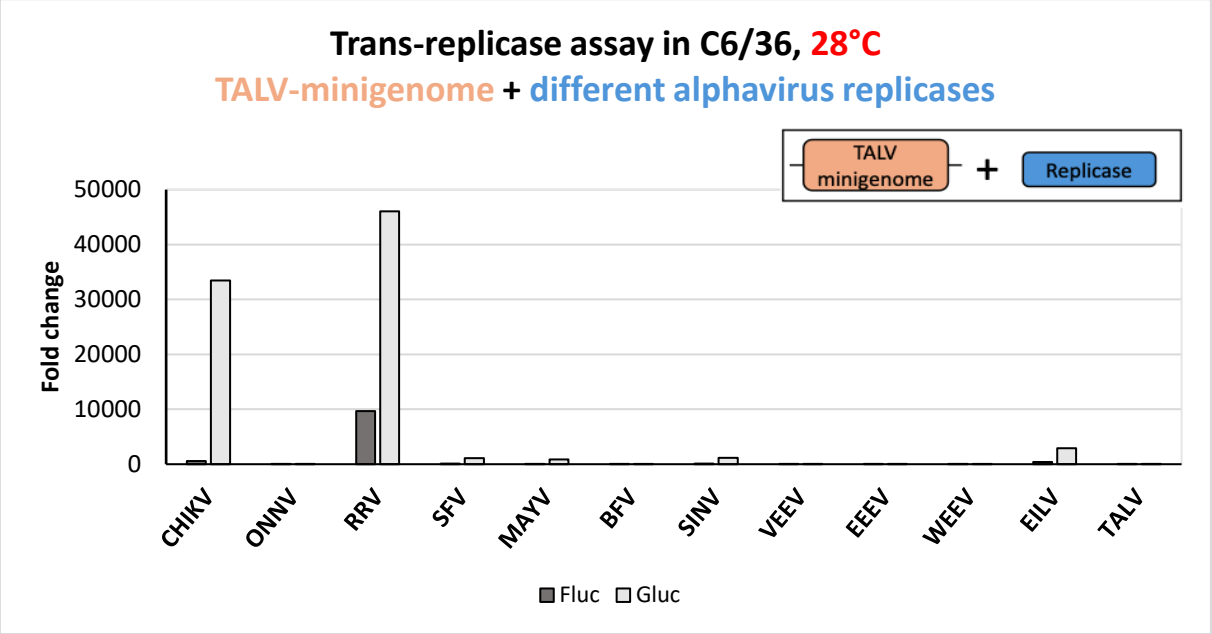

Figure 16. The data from the trans-replicase assay showing the compatibility of the TALV minigenome in C6/36 cells at 28°C. Column graphs show the efficiency of alphaviral replicases in replicating and transcribing TALV minigenomes, measured by FLuc and Gluc luciferase reporter activity. The x-axis indicates the different minigenomes and replicase combinations. Fold change (y-axis) represents the ratio of RLU in cells transfected with an active viral replicase (wt) versus the control cells transfected with a polymerase-inactive (GAA) variant.

Testing of TALV replicases and different alphavirus minigenomes in the C6/36 cells

The reverse assay, TALV replicase compatibility with various alphaviral minigenomes, was conducted to further evaluate phylogenetic distances between TALV and members of the *Alphavirus* genus.

As illustrated in Figure 17, the TALV replicase efficiently replicated the EILV minigenome but showed low activity against alphaviral minigenomes, except RRV. Its minigenome was recognised by TALV replicase, indicating that the TALV nsPs possess the capacity to recognise CSEs and RSEs of the RRV minigenome to drive the replication. Nevertheless, TALV replicase showed extreme specificity for its respective minigenome, indicating a dependence on TALV-type *cis*-acting elements or on unidentified requirements.

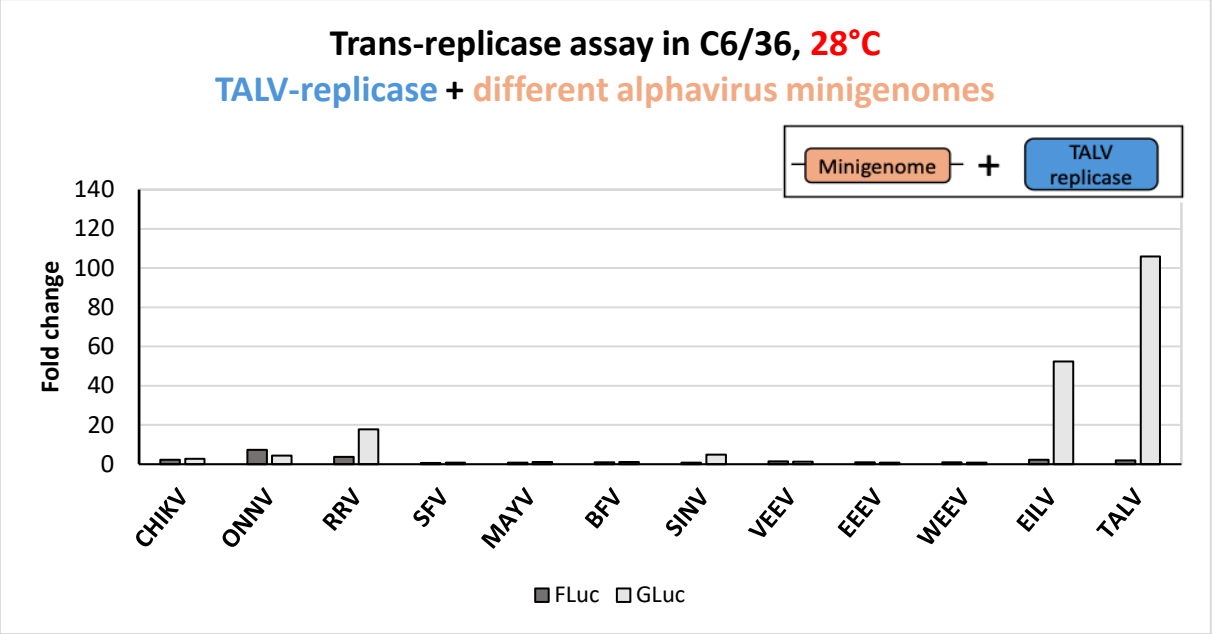

Figure 17. The data from the trans-replicase assay showing the compatibility of TALV replicase in C6/36 cells at 28°C. Column graphs show the efficiency of TALV replicases in replicating and transcribing alphaviral minigenomes, measured by FLuc and Gluc luciferase reporter activity. The x-axis indicates the different minigenomes and replicase combinations. Fold change (y-axis) represents the ratio of RLU in cells transfected with an active viral replicase (wt) versus the control cells transfected with a polymerase-inactive (GAA) variant.

Split trans-replicase assay of TALV, CHIKV, SINV and EILV constructs in the C6/36 cells

A split trans-replicase assay was used to evaluate interactions between TALV replicase components. The two-component systems usually split replicase functions onto separate P123 and nsP4 plasmids. However, in the study, TALV replicase functionality was split between a polymerase-inactive replicase (nsP1234-GAA) and a functional nsP4 plasmids. To test this assay, the TALV minigenome was co-transfected with different combinations of these two plasmids (Figure 18).

The TALV polyprotein compatibility assay combines TALV nsP1234-GAA with nsP4 from related alphaviruses to test how well the TALV polyprotein interacts with heterologous polymerases. Conversely, the reverse setup assesses TALV polymerase compatibility by pairing a functional TALV nsP4 plasmid with polyproteins from the same related viruses.

As shown in Figure 18, the wt TALV replicase was, expectedly, the most efficient at replicating its respective minigenome. However, TALV replication can also function to some extent using components from other alphaviruses. In the polyprotein compatibility assay, the TALV

nsP1234-GAA showed clear cross-compatibility by successfully recruiting nsP4 from both SINV and EILV. In contrast, pairing it with CHIKV nsP4 resulted in minimal activity.

The reverse polymerase compatibility assay did not show the same flexibility as the polyprotein setup. In this case, only the EILV nsP1234-GAA polyprotein was able to form a functional complex with TALV nsP4. These findings suggest that while TALV shares conserved features with alphaviruses from the SFV complex and SINV, the physical interaction between the TALV nsP4 polymerase and the nsP1234 scaffold relies on highly specific molecular determinants.

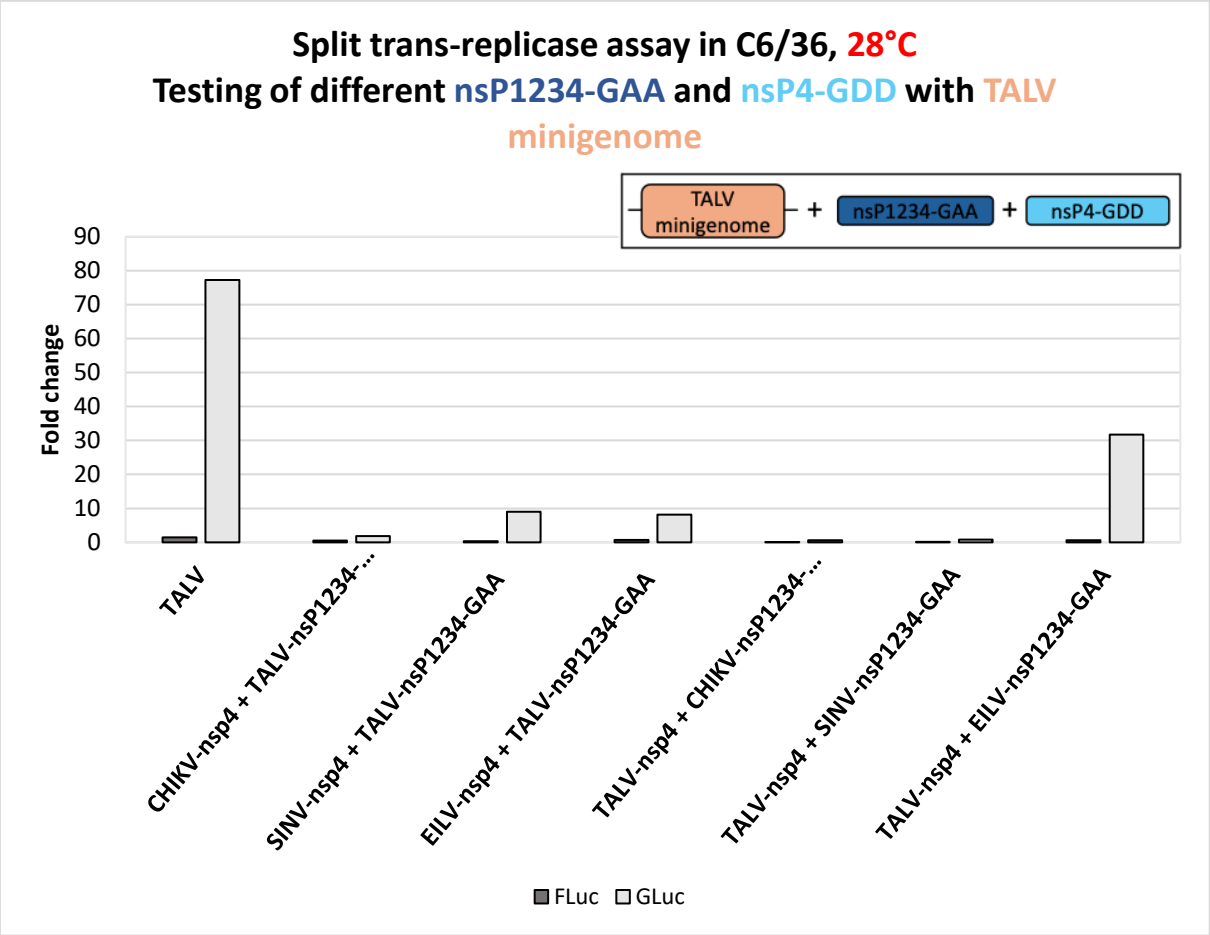

Figure 18. The data from the split trans-replicase assay in C6/36 cells at 28°C. Column graphs compare the replication and transcription efficiency of the TALV minigenome across different alphaviral nsP1234-GAA and nsP4 combinations, measured via FLuc and GLuc reporter activity. The x-axis indicates the specific polyprotein-polymerase combinations tested. Fold change (y-axis) represents the ratio of RLU in cells transfected with an active viral replicase (wt) versus the control cells transfected with a polymerase-inactive (GAA) variant. The combination of the TALV minigenome and TALV replicase wt serves as the positive control and is labelled "TALV" on the x-axis.

These findings suggest that the EILV and TALV replicase components are functionally compatible, consistent with their closer evolutionary relationship. In contrast, data reveal a clear functional asymmetry in the compatibility with SINV components. While SINV nsP4

efficiently recognises the TALV nsP1234-GAA, the reverse setup fails. TALV polymerase cannot successfully pair with SINV polyprotein. This asymmetry may indicate that the TALV polymerase relies on specialised molecular interactions with its polyprotein that are not conserved in more distantly related alphaviruses, such as SINV.

Overall, the experimental data confirm that the insect TALV trans-replicase assay is functional in C6/36 cells at 28°C. However, the TALV baseline replication efficiency varied across experiments, highlighting a need for further optimisation of the assay. The cross-compatibility results reflect TALV's phylogenetic relations to EILV, SINV, and the SFV complex. Ultimately, the split trans-replication assay results demonstrated that the TALV polymerase exhibits high specificity for its own polyprotein, enabling maximum minigenome replication.

2.3.3 Future perspective

The first developed trans-replicase assays were crucial for understanding TALV replication capacity and for determining optimal experimental setups. The next step in the study of the mammalian model will be to test it in different mammalian cell lines at 28°C and to incubate for longer than 24 hours. For the insect model, experiments must be conducted using the *Culex decens* cell line to identify the cause of TALV replication level variability and to discover unidentified host-specific requirements. To optimise the assay, replacing the minigenome promoter and testing in different insect cell lines may provide valuable data.

The trans-replicase assays confirmed that TALV occupies a well-defined phylogenetic position: closest to EILV, and basal to the WEEV complex. Critically, the split trans-replicase assay provided valuable insight into the conservation levels of alphavirus replicase components separately. Hence, studying the TALV's basal position might improve understanding of the evolutionary transition that enabled alphaviruses to move from insect-specific vectors to a wide range of hosts.

The future direction of this study will focus on further developing the trans-replicase assay across a wide range of cell lines and testing conditions. Furthermore, it will be possible to generate a TALV infectious complementary DNA (cDNA) clone and perform viral rescue to recover a replication-competent virus. This system will allow targeted mutations to investigate the roles of viral proteins and *cis*-acting elements, as well as to assess the contribution of host factors to alphavirus replication.

SUMMARY

This study represents a first step toward exploring the understudied TALV by successfully developing trans-replicase assays for both mammalian (U2OS) and insect (C6/36) cell lines. The host-specific plasmid systems were successfully constructed, and the functionality of trans-replicase assays was proven. TALV replication was found to be temperature-sensitive, with higher efficiency at 28°C than at 37°C, reflecting adaptation to its insect hosts. Strong compatibility was observed between TALV and EILV, supporting their close evolutionary relationship. TALV was also found to be compatible with SINV and members of the SFV complex (CHIKV, RRV), indicating that *cis*-acting elements are conserved across these alphaviruses. Importantly, TALV replicase exhibited extreme specificity for its own and EILV replicase components, as well as for the minigenome. These findings may imply that phylogenetically, TALV is the sister lineage of EILV and basal to the WEEV complex. Ultimately, TALV replication is functional but host-restricted, which may stem from either a narrow host range or dependence on yet-unidentified host factors. These results provide a foundation for future functional analysis of TALV.

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