

# **Rescue of Tomato spotted wilt tospovirus entirely from cDNA clones, establishment of the first reverse genetics system for a segmented (-)RNA plant virus**

Mingfeng Feng<sup>a</sup>, Ruixiang Cheng<sup>a</sup>, Minglong Chen<sup>a</sup>, Rong Guo<sup>a</sup>, Luyao Li<sup>a</sup>, Zhike Feng<sup>a</sup>, Jianyan Wu<sup>a</sup>, Li Xie<sup>b</sup>, Jian Hong<sup>b</sup>, Zhongkai Zhang<sup>c</sup>, Richard Kormelink<sup>d</sup> and Xiaorong Tao<sup>a,1</sup>

<sup>a</sup>Department of Plant Pathology, Nanjing Agricultural University, Nanjing 210095, P. R. China; <sup>b</sup>Analysis Center of Agrobiological Sciences, Zhejiang University, Hangzhou 317502, P. R. China; <sup>c</sup>Yunnan Provincial Key Laboratory of Agri-Biotechnology, Institute of Biotechnology and Genetic Resources, Yunnan Academy of Agricultural Sciences, Kunming, Yunnan 650223, P. R. China; <sup>d</sup>Laboratory of Virology, Department of Plant Sciences, Wageningen University, 6708PB Wageningen, The Netherlands

Author contributions: M.F., Z.F. and X.T. conceived and designed the experiments and H.J., Z.Z. and R. K. provided input. M.F., R.C., M.C., G.R., L.L., Z.F., J.W., and X.L. performed the experiments. M.F., R. K. and X.T. wrote the manuscript.

The authors declare no conflict of interest.

<sup>1</sup>To whom correspondence should be addressed. Email: [taoxiaorong@njau.edu.cn](mailto:taoxiaorong@njau.edu.cn)

## **Running title**

Establishment of a reverse genetics system for TSWV

## Abstract

The group of negative strand RNA viruses (NSVs) includes not only dangerous pathogens of medical importance but also serious plant pathogens of agronomical importance. Tomato spotted wilt tospovirus (TSWV) is one of those plant NSVs that cause severe diseases on agronomic crops and pose major threats to global food security. Its negative-strand segmented RNA genome has, however, always posed a major obstacle to molecular genetic manipulation. In this study, we report the complete recovery of infectious TSWV entirely from cDNA clones, the first reverse genetics (RG) system for a segmented plant NSV. First, a replication and transcription competent mini-genome replication system was established based on 35S-driven constructs of the S<sub>(-)</sub>-genomic (g) or S<sub>(+)</sub>-antigenomic (ag) RNA template, flanked by a 5' Hammerhead and 3' Ribozyme sequence of Hepatitis Delta virus, a nucleocapsid (N) protein gene and codon-optimized viral RNA dependent RNA polymerase (RdRp) gene. Next, a movement competent mini-genome replication system was developed based on M<sub>(-)</sub>-gRNA, which was able to complement cell-to-cell and systemic movement of reconstituted ribonucleoprotein complexes (RNPs) of S RNA replicon. After further optimization, infectious TSWV and derivatives carrying eGFP reporters were successfully rescued *in planta* via simultaneous expression of full-length cDNA constructs coding for S<sub>(+)</sub>-agRNA, M<sub>(-)</sub>-gRNA and L<sub>(+)</sub>-agRNA. Viral rescue occurred in the additional presence of various viral suppressors of RNAi, but TSWV NSs interfered with the rescue of genomic RNA. The establishment of a RG system for TSWV now allows detailed molecular genetic analysis of all aspects of tospovirus life

cycle and their pathogenicity.

**Key words:** Reverse genetics system, Tomato spotted wilt tospovirus, negative-strand RNA virus, tripartite RNA genome, mini-replicon, genome-length infectious cDNA clones

## Significance

For many different animal-infecting segmented negative-strand viruses (NSVs), a reverse genetics system has been established that allows the generation of mutant viruses to study disease pathology and the role of *cis*- and *trans*-acting elements in the virus life cycle. In contrast to the relative ease to establish RG systems for animal-infecting NSVs, establishment of such system for the plant-infecting NSVs with a segmented RNA genome so far has not been successful. Here we report the first reverse genetics system for a segmented plant NSV, the Tomato spotted wilt tospovirus, a virus with a tripartite RNA genome. The establishment of this RG system now provides us with a new and powerful platform to study their disease pathology during a natural infection.

## Introduction

Negative-stranded RNA viruses (NSVs) present of a large group of viruses that include well known members of medical importance such as Ebola (EBOV), Rabies (RV), Influenza A (FLUAV) and Rift valley fever virus (RVFV) (1, 2). Infections with these viruses may cause considerable morbidity and mortality in humans and form an important burden on national health care budgets. The group also contains plant viruses of agronomical importance such as Tomato spotted wilt virus (TSWV) and Rice stripe virus (RSV) that cause severe diseases on agronomic crops and pose major threats to global food security (3-10).

Tospoviruses belong to the NSV with a segmented (tripartite) RNA genome and rank among the most devastating plant viruses worldwide (11, 12). They are classified in the family of *Tospoviridae* within the order *Bunyavirales* (13). TSWV is the type member of the only genus *Orthotospovirus*, in the family of *Tospoviridae* (11, 13). TSWV has very broad host range infecting more than one thousand plant species over 80 families (14) and is transmitted by thrips in a persistent, propagative manner (6, 9, 15, 16). Crops losses due to this virus have been estimated more than one billion dollars annually (7, 14).

TSWV consists of spherical, enveloped virus particles (80-120 nm) that contain a tripartite genome consisting of a large- (L), medium- (M), and small-sized (S) RNA segment (11). The L segment is of entire negative polarity, whereas the S and M segments are ambisense. The L segment encodes the viral RNA-dependent RNA polymerase (RdRp, ~330 kDa) that is required for viral RNA replication and mRNA

transcription (17, 18). The viral (v) strand of the M segment encodes the precursor to the glycoproteins (Gn and Gc, with n and c referring to the amino- and carboxy-terminal end of the precursor, respectively) in the negative sense and a nonstructural protein (NSm) in the positive sense. The glycoproteins are required for particle maturation and are presented as spikes on the surface of the virus envelope membrane (19, 20). They also play a major role as determinants for thrips vector transmission (21). The NSm plays pivotal roles in cell-to-cell and long distance movement of TSWV (22-26). The vRNA of the S segment codes for the nucleocapsid protein (N) in the negative sense and a nonstructural protein (NSs) in the positive sense. The N protein participates in the formation of ribonucleoprotein complexes (RNPs) (27-29) and is required for viral intracellular movement (30, 31). The NSs protein functions as a RNA silencing suppressor to defend against the plant innate immunity system (32-34), and triggers a defence response and concomitant programmed cell-death mediated by the dominant resistance gene *Tsw* from *Capsicum chinense* (35-37).

As a virus documented for almost a century (7, 38), TSWV has served as an important model for studying the molecular biology of tospovirus and other plant NSVs with segmented genomes (6-8, 11). However, its negative-strand tripartite RNA genome has posed a major obstacle to genetic manipulation of the virus. To initiate an infection cycle with this virus requires at least RNPs, the minimal infectious units that consist of viral RNA encapsidated by the N protein and associated with a few copies of the viral RNA-dependent RNA polymerase (6, 11). TSWV RNPs can be

mechanically transferred from infected to healthy plants, however, transmission by thrips requires RNPs to be enveloped and spiked with the glycoproteins (21).

The first animal-infecting and related counterpart of TSWV with a segmented RNA genome to be rescued entirely from cDNA was from Bunyamwera virus in 1996 (39). Following this study, soon other segmented NSV were rescued from plasmid DNA. The influenza virus, containing a genome of eight RNA segments, was recovered in 1999 (40), while the first Arenavirus, with a bipartite RNA genome, was recovered in 2006 (41). Just recently, the first nonsegmented plant NSVs from the *Mononegavirales* have been rescued, *i.e.* the *Sonchus* yellow net nucleorhabdovirus (SYNV) (42, 43). A recent study also reported on the establishment of a TSWV S RNA-based mini-replicon in yeast (44), but in contrast to replication, no transcriptional activity was observed.

In contrast to the relative ease to establish RG systems for animal-infecting NSVs, reconstitution of infectious RNPs *in planta* for the plant-infecting viruses with a segmented RNA genome seems particularly difficult. Not only have all DNA constructs to be delivered into one and the same plant cell but for TSWV the RdRp is exceptionally large (~330 kDa) compared to the RdRp of most other related bunyaviruses (~240-260 kDa) and to typical open reading frames (ORFs) from the plant genome. Expression of such a large protein gene may not only be very inefficient but mRNA transcripts resulting from RNA polymerase II transcription of 35S promoter-constructs in the nucleus may also face splicing of cryptic splicing sites. Moreover, achieving proper ratios of all three genome segments in plant cells is not

easily and consistently achieved by agrobacterium-mediated delivery of several constructs, which will affect the outcome of each individual experiment. All these obstacles may hamper the construction of a reverse genetics system for TSWV in plants.

In this study, we report the complete recovery of infectious TSWV entirely from cDNA clones in plants, the first reverse genetics system for a segmented plant NSV. The establishment of this system presents the start of a new research era for TSWV and provides us an entirely new and powerful platform to study the basic principles of the tospovirus life cycle and viral pathogenicity.

## Results

### Development of a TSWV S<sub>(-)</sub>-genomic RNA mini-replicon system in *Nicotiana benthamiana*

Prior to the rescuing of TSWV entirely from cDNA clones, a mini-replicon system based on the S RNA-template was established. To this end, a DNA copy of the TSWV S<sub>(-)</sub>-genomic RNA (gRNA) was cloned and flanked with a self-cleaving hammerhead (HH) ribozyme at the 5'-terminus and a hepatitis delta virus (HDV) ribozyme at the 3'-terminus. For visual monitoring, quantification purposes, and discrimination between primary and secondary genome transcription, the NSs and N genes were replaced with mCherry and eGFP, respectively (Fig. 1A). The resulting S<sub>(-)</sub> mini-replicon reporter was cloned in a binary vector pCB301 downstream a double 35S promoter (2×35S) and denoted 35S:SR<sub>(-)</sub>mCherry&eGFP (Fig. 1A), or a T7 promoter

and denoted T7: SR<sub>(-)</sub>mCherry&eGFP (Fig. S1A). The RdRp and N ORFs were amplified from cDNA of TSWV infected tissue and cloned into pCambia 2300 binary vector downstream a double 35S promoter. Binary vector constructs of the RdRp, N and four viral RNA silencing suppressor genes (VSRs: NSs from TSWV, P19 from Tomato bushy stunt tobravirus (TBSV), HcPro from Tobacco etch potyvirus (TEV) and  $\gamma$ b from Barley yellow mosaic hordeivirus (BYMV)) were agroinfiltrated in *N. benthamiana* either with 35S:SR<sub>(-)</sub>mCherry&eGFP or with T7:SR<sub>(-)</sub>mCherry&eGFP and a 35S-driven T7 RNA polymerase gene construct and next monitored for eGFP fluorescence. However, during repeated experiments no eGFP fluorescence was observed from 35S:SR<sub>(-)</sub>mCherry&eGFP nor T7:SR<sub>(-)</sub>mCherry&eGFP (Fig. S1B and C).

The possibility of failures to establish a mini-replicon system for TSWV could be due to low (unstable) expression of the TSWV RdRp protein, therefore, the codon usage of the RdRp gene was optimized for *N. benthamiana* and potential intron splicing sites were removed. The optimized RdRp gene (RdRp<sub>opt</sub>) was cloned in a binary, 35S-driven expression vector and next, again agroinfiltrated in *N. benthamiana* leaves together with binary expression constructs of the N gene, the four VSR gene constructs and the 35S:SR<sub>(-)</sub>mCherry&eGFP mini-replicon reporter. At 5 days post infiltration (dpi), expression of the reporter genes was analyzed by monitoring for mCherry and eGFP fluorescence in the *N. benthamiana* leaves (Fig. 1A and B). Whereas no fluorescence was observed in the controls, *i.e.* leaves agroinfiltrated with 35S:SR<sub>(-)</sub>mCherry&eGFP alone or co-expressing 35S:SR<sub>(-)</sub>mCherry&eGFP with RdRp<sub>opt</sub> or N only, eGFP and mCherry fluorescence was consistently observed in leaves



agroinfiltrated with 35S:SR(-)mCherry&eGFP and both N and RdRp<sub>opt</sub> (Fig. 1C). This was confirmed by Western immunoblot analysis (Fig. 1D).

Northern blot analysis showed that both the genomic RNA and anti-genomic RNA of the SR(-)mCherry&eGFP mini-replicon were detected in the leaves that co-expressed both N and RdRp (here after RdRp represent optimized RdRp) at 5 dpi, but not in the leaves co-expressing RdRp or N only (Fig. 1E). In addition, using an anti-sense eGFP probe genome length S RNA subgenomic-sized RNA, likely presenting eGFP transcripts, were detected (Fig. 1E, upper panel). A time course analysis showed that eGFP and mCherry fluorescence in *N. benthamiana* leaves was visible from 3 dpi onwards and gradually increased to 12 dpi (Fig. S2A). This was also confirmed by immunoblot analysis (Fig. S2B).

Altogether the results indicated that in *N. benthamiana* the 35S replicon transcript SR(-)mCherry&eGFP was properly processed by the HH and RZ, and next used by the RdRp as template for primary transcription of mRNA<sup>eGFP</sup>, as well replicated into SR(-)mCherry&eGFP for secondary transcription of mRNA<sup>mCherry</sup>. Furthermore, the codon-optimized RdRp clearly exhibited full functionality in supporting viral genome transcription and replication, while the wild type RdRp for unknown reasons didn't. When the T7:SR(-)mCherry&eGFP mini-replicon reporter was co-expressed with T7 RNA polymerase, RdRp, N and four VSRs in *N. benthamiana* leaves, somewhat unexpected, no mCherry or eGFP fluorescence was detected (Fig. S1C and D).

**The optimization of the concentration of N and RdRp, and VSRs on TSWV**

## 199 **SR<sub>(-)</sub>mCherry&eGFP mini-replicon**

200 Having established a TSWV S<sub>(-)</sub>-gRNA based mini-replicon system in *N.*  
 201 *benthamiana*, attempts were made to further optimize the system. To this end, binary  
 202 constructs of the TSWV S<sub>(-)</sub> mini-replicon were agroinfiltrated into *N. benthamiana* in  
 203 the additional presence of varying amounts of N and RdRp gene expression constructs.  
 204 During these experiments the amounts of *Agrobacterium* carrying the N expression  
 205 construct were increased (from OD<sub>600</sub> 0.2 to 0.8) while the *Agrobacterium* harboring  
 206 the RdRp expression construct was kept fixed at OD<sub>600</sub> 0.2, and vice versa. The results  
 207 showed highest eGFP reporter gene expression from the 35S:SR<sub>(-)</sub>mCherry&eGFP  
 208 mini-replicon when *Agrobacterium* suspensions bearing N and RdRp expression  
 209 constructs were both infiltrated at OD<sub>600</sub> 0.2. When *Agrobacterium* harboring either N  
 210 or RdRp was infiltrated onto *N. benthamiana* leaves at OD<sub>600</sub>>0.4 the expression of  
 211 eGFP from the S<sub>(-)</sub>-mini-replicon greatly decreased (Fig. 2 A, B and C). Furthermore,  
 212 at high concentrations (OD<sub>600</sub> > 0.6) of *Agrobacterium*, the visible cell death was  
 213 triggered in the infiltrated leaves (data not shown).

214 Next, in a similar approach and using the optimized setting, the effects of VSRs  
 215 on the replication and transcription of the SR<sub>(-)</sub>mCherry&eGFP mini-replicon were  
 216 investigated. Without the addition of VSRs, mCherry and eGFP fluorescence was only  
 217 observed in a small number of cells, but these numbers increased in the addition of  
 218 TSWV NSs and/or the three VSRs P19, HcPro and γb. The largest number of cells  
 219 showing eGFP expression from the S<sub>(-)</sub> mini-replicon, as monitored by fluorescence,  
 220 were obtained when all four VSRs were added (Fig. 2D and E). These observations

were further confirmed by immunoblot assays (Fig. 2E).

## The role of *cis*-acting sequences in transcription and replication of the TSWV

### SR<sub>(-)</sub>mCherry&eGFP replicon

Using the optimized SR<sub>(-)</sub>mCherry&eGFP mini-replicon system, the role of the 5'-untranslated region (5'-UTR), 3'-UTR and the non-coding A/U-rich intergenic region (IGR) between NSs and N genes (45, 46) in replication-transcription was examined. To this end, SR<sub>(-)</sub>mCherry&eGFP derivatives were made from which the 5'-UTR, IGR or 3'-UTR, respectively, were deleted and next tested on transcription-replication using the mini-replicon assay (Fig. S3A). No eGFP and mCherry fluorescence was observed when the 5'-UTR or 3'-UTR of SR<sub>(-)</sub>mCherry&eGFP was removed. However, eGFP reporter expression could still be detected when the IGR of SR<sub>(-)</sub>mCherry&eGFP was deleted (Fig. S3B). Immunoblot analysis confirmed the expression of eGFP from SR<sub>(-)</sub>mCherry&eGFPΔIGR (ΔIGR), and lack of expression from SR<sub>(-)</sub>mCherry&eGFPΔ5'UTR (Δ5'UTR) and SR<sub>(-)</sub>mCherry&eGFPΔ3'UTR (Δ3'UTR) (Fig. S3C). To analyze whether the lack of eGFP expression was a matter of translation or transcription-replication, samples from infiltrated *N. benthamiana* were collected and analyzed by Northern blot. The results showed that for SR<sub>(-)</sub>mCherry&eGFPΔ5'UTR (Δ5'UTR) and SR<sub>(-)</sub>mCherry&eGFPΔ3'UTR (Δ3'UTR), weak signals of genomic RNA could be detected but they are not similar to the signal strength obtained for the genome length and mRNA molecules as seen with the SR<sub>(-)</sub>mCherry&eGFP replicon, while anti-genomic RNAs could not be detected (Fig. S3D). For SR<sub>(-)</sub>mCherry&eGFPΔIGR (ΔIGR) both RNA strands were detected (Fig. S3D), suggesting that IGR is not essential for viral RNA synthesis,

while no signal is obtained for the mRNA length molecules as seen with the SR<sub>(-)</sub>mCherry&eGFP replicon. Taken together, these findings suggest that the 5'-UTR and 3'-UTR play an essential role in viral transcription and replication of TSWV RNA segments.

### **Development of a TSWV S<sub>(+)</sub>-agRNA based mini-replicon system**

For many reverse genetics systems of NSV, mini-replicons have initially been established based on gRNA (vRNA). Here, we managed to develop a first system for TSWV based on antigenomic (ag)RNA (vcRNA). In order to analyze whether a system could be developed based on agRNA, a S<sub>(+)</sub>-agRNA mini-replicon was constructed similarly to the one based on S<sub>(-)</sub>-gRNA but in which the N gene was maintained and only the NSs gene was replaced by eGFP, denoted SR<sub>(+)</sub>eGFP (Fig. 3A). In analogy to the replicon assays with SR<sub>(-)</sub>mCherry&eGFP, *N. benthamiana* leaves were agro-infiltrated with binary expression constructs of SR<sub>(+)</sub>eGFP, four VSRs and either N or RdRp separately or together, respectively, and monitored for eGFP fluorescence. Whereas eGFP fluorescence, resulting from primary transcription of the replicon transcript by viral RdRp, was not detected when SR<sub>(+)</sub>eGFP was expressed alone or in the additional presence of N, eGFP fluorescence was observed when SR<sub>(+)</sub>eGFP was co-expressed with both RdRp and N, but also when SR<sub>(+)</sub>eGFP was co-expressed with RdRp alone (Fig. 3B). This strongly indicated that a certain (residual) amount of SR<sub>(+)</sub>eGFP transcripts, resulting from 35S transcription, did not become fully processed by the HH and RZ and remained functional in translation, thereby giving rise to N

protein. This was confirmed by Western immunoblot analysis (Fig. 3C). Northern blot analysis furthermore showed that samples from the replicon assays performed in the presence of RdRp and N or RdRp alone, besides eGFP mRNA transcripts, also contained agRNA and gRNA of SR<sub>(+)</sub>eGFP, indicating the occurrence of replication (Fig. 3D). Altogether, these results demonstrate that the N protein can also be expressed from the SR<sub>(+)</sub>eGFP replicon to support its transcription and replication. This provides an attractive alternative to the S<sub>(-)</sub>-gRNA based mini-replicon as additional binary expression constructs for N do not have to be supplied anymore.

## **Development of a M<sub>(-)</sub>-gRNA based mini-replicon for cell-to-cell movement of TSWV in *N. benthamiana***

As a first step towards development of a reverse genetics system to rescue TSWV virus entirely from cDNA, a movement competent mini-replicon was also established. To this end, a TSWV M<sub>(-)</sub>-gRNA based mini-replicon was constructed, similar as the ones made for S<sub>(-)</sub> and S<sub>(+)</sub>. Within this construct the NSm cell-to-cell movement protein gene was maintained but the GP ORF was exchanged for eGFP, resulting in a mini-replicon designated as MR<sub>(-)</sub>eGFP (Fig. 4A). After *Agrobacterium*-mediated delivery into *N. benthamiana*, no eGFP fluorescence was observed in leaves containing the MR<sub>(-)</sub>eGFP replicon with RdRp or N. However, in the presence of both RdRp and N, eGFP fluorescence was observed in many cells that connected each other (Fig. 4B and C). In comparison to the eGFP fluorescence always expressed in a single plant cells from SR<sub>(-)</sub>mCherry&eGFP or SR<sub>(+)</sub>eGFP reporter, the results suggested that

the MR<sub>(-)</sub>eGFP mini-replicon has moved from cell-to-cell in *N. benthamiana* leaves. Northern blot analysis confirmed the synthesis of gRNA, agRNA and (subgenomic-length) eGFP mRNA transcripts of the MR<sub>(-)</sub>eGFP replicon in the presence of both RdRp and N, but not with RdRp or N only (Fig. 4D).

To further substantiate the findings on possible cell-to-cell movement of the MR<sub>(-)</sub>eGFP mini-replicon, a stop codon was introduced immediately downstream the start codon of NSm and the construct designated MR<sub>(-)</sub>eGFP&NSmMut (Fig. 4A). When the MR<sub>(-)</sub>eGFP&NSmMut replicon was delivered and co-expressed with RdRp and N in *N. benthamiana* leaves, eGFP fluorescence was only detected in a single cells (Fig. 4B). As expected, Western immunoblot analysis confirmed the production of eGFP protein in leaves containing the MR<sub>(-)</sub>eGFP&NSmMut replicon, and in significantly lower amounts compared to the MR<sub>(-)</sub>eGFP replicon (Fig. 4C).

# **Establishment of the systemic infection of M<sub>(-)</sub>- and S<sub>(+)</sub>-mini-replicon reporters by co-expression of full-length antigenomic L<sub>(+)</sub> in *N. benthamiana***

With the establishment of S (g/ag)RNA-based mini-replicon systems, and a movement-competent M gRNA-based mini-replicon, we set out to construct full length genomic cDNA clones of L<sub>(-)</sub>, M<sub>(-)</sub> and S<sub>(-)</sub>, flanked by HH and HDV at 5'- and 3'-terminus, as a first step towards the rescue of TSWV entirely from cDNA clones. At the same time, similar constructs were made for the anti-genomic L<sub>(+)</sub>, M<sub>(+)</sub> and S<sub>(+)</sub>. However, attempts to recover infectious TSWV from *N. benthamiana* after agrobacterium-mediated delivery of all binary expression constructs of L<sub>(-)</sub>, M<sub>(-)</sub>, S<sub>(-)</sub>

together with N, RdRp and four VSRs, but also with the anti-genomic L<sub>(+)</sub>, M<sub>(+)</sub> and S<sub>(+)</sub> constructs, all failed (Table 1).

Since MR<sub>(-)eGFP</sub> was earlier shown to be movement competent, it was next investigated whether the M<sub>(-)</sub>- and S<sub>(-)</sub>-minigenomes moved into the same plant cell in the presence of both RdRp and N. Upon co-expression of MR<sub>(-)mCherry</sub>, SR<sub>(-)eGFP</sub>, RdRp and N in *N. benthamiana* leaves, expression of both mCherry and eGFP from the MR<sub>(-)mCherry</sub> and SR<sub>(-)eGFP</sub> mini-replicons, respectively, could be discerned. However, the foci of mCherry fluorescence were separate from those showing eGFP fluorescence (Fig. S4). Previously, ectopic expression of Tobacco crinkle virus (TCV) RdRp was reported to cause superinfection exclusion, and prevented the entry of progeny virus into the original cell expressing the RdRp (47). Ectopic expression of TSWV RdRp and N would possibly cause superinfection exclusions and block intercellular movement of SR<sub>(-)eGFP</sub> into cells containing MR<sub>(-)mCherry</sub>. To avoid that, a new strategy was employed in which RdRp and N were expressed from viral agRNAs. To this end, a construct was made of the full-length L agRNA containing the optimized RdRp and flanked by the HH and HDV ribozymes, denoted L<sub>(+)opt</sub> (Fig. 5A). To test the expression and functionality of RdRp from this construct, L<sub>(+)opt</sub> was co-expressed with the SR<sub>(-)mCherry&eGFP</sub> mini-replicon, N and VSRs in *N. benthamiana* leaves. The results showed clear eGFP and mCherry fluorescence and indicated that L<sub>(+)opt</sub> was able to support SR<sub>(-)mCherry&eGFP</sub> transcription and replication (Fig. S5A). Furthermore, L<sub>(+)opt</sub> was also able to support the replication and transcription of the SR<sub>(+)eGFP</sub> mini-replicon, without the additional ectopic expression of N (Fig. S5B),

and of the movement competent MR<sub>(-)eGFP</sub> (Fig. S5C).

In a next experiment L<sub>(+)opt</sub> was co-expressed with MR<sub>(-)mCherry</sub>, SR<sub>(+)eGFP</sub> and four VSRs in *N. benthamiana* and plants analyzed for a systemic infection (Fig. 5A). At 6 dpi, mCherry and eGFP fluorescence were detected in the locally agroinfiltrated *N. benthamiana* leaves and in which some foci were found to express mCherry and eGFP together (Fig. S6A). At 15 dpi, necrotic symptoms became visual in systemic leaves of *N. benthamiana* (Fig. 5B, a and c). Using a handheld UV lamp, a clear eGFP fluorescence was also observed in those leaves (Fig. 5B, b and d). The eGFP signal was detected in 24 out of 30 agro-infiltrated *N. benthamiana* plants (Table 1). Both eGFP and mCherry fluorescence were detected in veins/stems and systemic leaves under a fluorescence microscope (Fig. 5B, e). The systemic infection of *N. benthamiana* with SR<sub>(+)eGFP</sub>, MR<sub>(-)mCherry</sub> and L<sub>(+)opt</sub> was further confirmed by RT-PCR analysis (Fig. S6B).

### Recovery of infectious TSWV from the full-length cDNA clones

Based on the establishment of a systemic infection after *Agrobacterium*-mediated delivery of replicons SR<sub>(+)eGFP</sub>, MR<sub>(-)mCherry</sub> and L<sub>(+)opt</sub>, we next generated a full-length construct for S agRNA without any reporter gene, designated as S<sub>(+)</sub> and co-expressed it with replicon constructs L<sub>(+)opt</sub>, M<sub>(-)</sub> and four VSRs in *N. benthamiana* leaves. However, and surprisingly, no infectious TSWV was recovered from systemic leaves of *N. benthamiana* that were infiltrated with these constructs (Table 1). To find out whether this was due to failure of S<sub>(+)</sub>, we next examined whether S<sub>(+)</sub> was able to



establish a systemic infection in combination with the functional MR<sub>(-)eGFP</sub> and L<sub>(+)opt</sub> constructs. When L<sub>(+)opt</sub>, MR<sub>(-)eGFP</sub>, S<sub>(+)</sub> and three VSRs (P19-HcPro- $\gamma$ b) were co-expressed in *N. benthamiana* leaves, eGFP fluorescence was visible at 18 dpi in systemic leaves of *N. benthamiana*, although not as efficient as in the case with the SR<sub>(+)eGFP</sub> replicon, since only 7 out of 60 plants showed systemic infection (Fig. 5D and Table 1). RT-PCR analysis confirmed the systemic infection with S<sub>(+)</sub>, MR<sub>(-)eGFP</sub> and L<sub>(+)opt</sub> in those *N. benthamiana* (Fig. S6C). When L<sub>(+)opt</sub>, MR<sub>(-)eGFP</sub> and S<sub>(+)</sub> were co-expressed with four VSRs (P19-HcPro- $\gamma$ b and NSs) in *N. benthamiana* leaves, intriguingly, no eGFP fluorescence was observed in the systemic leaves (Table 1) suggesting that ectopic expression of NSs interfered with the rescue of virus from full length S<sub>(+)</sub>, MR<sub>(-)eGFP</sub> and L<sub>(+)opt</sub>.

Next, we tested the rescuing of virus from full length M<sub>(-)</sub>, SR<sub>(+)eGFP</sub> and L<sub>(+)opt</sub>. To this end, the constructs of L<sub>(+)opt</sub>, M<sub>(-)</sub> and SR<sub>(+)eGFP</sub> were delivered and co-expressed in *N. benthamiana* in the presence of either four (P19-HcPro- $\gamma$ b+NSs) or three (P19-HcPro- $\gamma$ b) VSRs. The results showed no eGFP fluorescence in systemic leaves of *N. benthamiana* at 15-50 dpi, indicating that M<sub>(-)</sub> was not able to complement and rescue the S<sub>(+)</sub>-mini-replicons into systemic leaves (Table 1). Northern blot analysis showed that neither gRNAs nor agRNAs were detected for M<sub>(-)</sub> while, in contrast, gRNAs and agRNAs were detected for S<sub>(+)</sub> (Fig. S7A and B). Earlier, the MR<sub>(-)eGFP</sub> mini-replicon was shown to replicate and transcribe (Fig. 4D). The only difference between M<sub>(-)</sub> and MR<sub>(-)eGFP</sub> mini-replicon was the GP gene, which was exchanged for eGFP in the second construct. Considering that primary M<sub>(-)</sub> transcripts were

produced in the nucleus by the 35S promoter, and putative splice sites were also predicted in the GP sequence (*SI Appendix, Table S2*), it was likely that primary M<sub>(-)</sub> transcripts were prone to splicing before sufficient replication of the mini-replicon and transcriptional-translational expression of the cell-to-cell movement protein gene could take place. For this reason, codon optimization was performed on the GP gene sequence in M<sub>(-)</sub>, leading to a new construct designated as M<sub>(-)opt</sub> (*Fig. 5A*). Upon co-expression of L<sub>(+)opt</sub>, M<sub>(-)opt</sub> and the SR<sub>(+)eGFP</sub> mini-replicon in *N. benthamiana* eGFP fluorescence was observed in systemic leaves (*Fig. 5C*). Fluorescence was observed in 27 out of 30 agroinfiltrated plants, and demonstrated that M<sub>(-)opt</sub> produced a functional and stable M genomic RNA, able to replicate and support systemic movement of S and L RNP molecules by its encoded NSm protein (*Table 1*). RT-PCR analysis further confirmed a systemic infection of *N. benthamiana* with SR<sub>(+)eGFP</sub>, M<sub>(-)opt</sub> and L<sub>(+)opt</sub> (*Fig. S6D*).

In a final experiment, aiming to rescue “wild type” TSWV entirely from cDNA clones, the binary constructs of L<sub>(+)opt</sub>, M<sub>(-)opt</sub> and S<sub>(+)</sub> were agroinfiltrated into *N. benthamiana* leaves together with three VSRs (P19-HcPro-γb). At 19 dpi, typical leaf curling was observed in systemic leaves from *N. benthamiana* plants (*Fig. 6A and Table 1*). Upon disease progression, plants started to exhibit a stunted phenotype between 19-30 dpi. When the experiment was repeated with a large batch of plants, a systemic infection was observed in 6 out of 60 plants (*Table 1*). Northern blot analyses on samples collected from systemically infected leaves showed the presence of gRNA and agRNA of S, M and L RNA segments (*Fig. 6B*), which was also

confirmed by RT-PCR (Fig. S8). Moreover, sequence analysis of the amplicons derived from the L and M RNA confirmed the presence of codon optimized RdRp and GP gene sequences (Fig. 6C). Immunoblot analysis on systemically infected leaf samples showed the presence of N, NSs, NSm, Gn and Gc proteins *N. benthamiana* (Fig. 6D), altogether indicating a successful systemic infection with rescued TSWV (rTSWV).

To demonstrate genuine virus particle rescue of rTSWV, samples were collected from newly infected systemic leaf tissues and subjected to transmission electron microscopy. As shown in Fig. 6E, typical enveloped and spherical virus particles were observed in rTSWV-infected tissue, altogether indicating that infectious TSWV (rTSWV) was successfully rescued from full-length cDNA clones of L<sup>(+)</sup>opt, M<sup>(-)</sup>opt and S<sup>(+)</sup>.

## Discussion

The establishment of a reverse genetics system for a segmented NSV basically requires two steps. The first one involves the *in vivo* reconstitution of transcriptionally active RNPs, often managed by development of a mini-genome replication system. The second step involves virus rescue entirely from full-length "infectious" cDNA clones, based on tools developed and optimized with the mini-genome replication system. In this study, we first successfully reconstituted infectious RNPs based on TSWV S<sup>(-)</sup>-gRNA and S<sup>(+)</sup>-agRNA after having optimized the sequence of RdRp. Next, a movement competent mini-genome replication system was developed based

on M<sub>(-)</sub>-gRNA, which was also able to complement and systemically rescue reconstituted S RNPs. In a third step, full length constructs were made for S<sub>(+)eGFP</sub>-agRNA, M<sub>(-)mCherry</sub>-gRNA and L<sub>(+)</sub>-agRNA, to directly accommodate for translation of (small amounts of) all three genomic (35S) transcripts into N, NSm and RdRp proteins, respectively, and leave out the additional need of ectopically expressed N and RdRp. *Agrobacterium*-mediated delivery of these constructs lead to a successful systemic infection of *N. benthamiana* with rTSWV carrying eGFP reporters. In a last step, the GP gene sequence of M<sub>(-)</sub> was optimized, that allowed the final rescuing of infectious rTSWV particles entirely from full-length cDNA clones in *N. benthamiana*.

The genomic RNAs of segmented NSVs possess neither a 5' cap-structure nor 3'-poly(A) tail (2, 48). Instead, their termini contain highly conserved sequences that show inverted sequence complementary and fold into a panhandle structure with a major role in RNA transcription and replication. Any additional nucleotide residues at those termini in the past have been shown to disrupt/affect transcription-replication of animal-infecting segmented NSVs (49). For this reason, the choice of plant promoter to generate the first primary full-length genomic RNA templates (mimicking authentic genomic RNA molecules) for initiating viral replication is one of the major and critical factors for the construction of a reverse genetics system for TSWV. For animal-infecting segmented NSVs, researchers in the past have been using various systems. One of the first strategies employed bacteriophage T7 promoter constructs co-expressed with a T7 RNA polymerase and later followed by the use RNA

polymerase I (Pol I) promoter constructs to generate the initial viral genome length RNA transcripts in mammalian cells (39, 40, 50-52). Unfortunately, attempts to establish the TSWV mini-replicon system based on the T7 promoter and T7 RNA polymerase strategy was unsuccessful (Fig. S1A, C and D). The activity of the Pol I promoter was shown to be species-dependent (53). Although a Pol I promoter has been reported from *Arabidopsis* (54, 55), while the transcription initiation +1 site is still not known. For *N. benthamiana* no Pol I promoter has been characterized yet. The 35S promoter, an RNA Pol II promoter, is well characterized and hence remains the only choice to establish a reverse genetics system for TSWV in plants. This is in contrast to reverse genetics of segmented NSVs in animal cells, where all viruses have been reconstituted after T7/Pol I driven production of primary viral RNA templates for replication. The Pol II promoter has been used to produce the initial viral RNA transcripts of an animal-infecting nonsegmented NSV (56). Recently, the 35S/Pol II promoter was also successfully employed to produce primary viral RNA template of the first non-segmented plant NSV reconstituted, the SYN RV rhabdovirus (42, 43). Here, we successfully deployed the 35S/Pol II promoter and two ribozymes at 5' and 3' ends of viral RNA sequences, to generate full length viral RNA transcripts that are recognized as initial/"authentic" RNA templates for TSWV replication and transcription by viral N and RdRp.

Besides the right promoter, the RdRp protein may present another bottleneck for the establishment of a reverse genetics system. Tospoviruses code for a single, unprocessed ~330 kDa RdRp from the 8.9 kb-sized L RNA (17, 18). The RdRp gene

sequence of TSWV was predicted to contain numerous intron splicing sites (*SI Appendix, Table S1*). Since the first animal segmented negative strand RNA virus was rescued in 1996 (39), numerous groups worldwide have attempted to construct a reverse genetics system for a tospovirus in plants. Here, it is shown that codon optimization and removal of potential intron splicing sites have been crucial for the expression of a functional RdRp of tospovirus from 35S-driven constructs *in planta* (Fig. 1B). While codon optimization may have contributed to increased protein expression levels, removal of predicted potential intron splicing sites from the RdRp genemay have helped to further stabilize and increase expression levels. After all, TSWV is known to replicate in the cytoplasm (2, 48), and its RdRp gene may not have been evolved to escape from the nuclear (pre-mRNA) splicing machinery. However, after nuclear transcription of the RdRp gene by the 35S promoter, any intron splicing site in the wild type RdRp transcript could thus be spliced and result in a truncated, non-functional RdRp.

Not only for RdRp, but also an optimized GP gene sequence turned out to be crucial to rescue a full length M RNA-based transcriptionally active RNP. Whereas the M<sub>(-)mCherry</sub> mini-replicon was able to establish a systemic infection in *N. benthamiana* when co-expressed with S<sub>(+)eGFP</sub> mini-replicon and L<sub>(+)opt</sub>, the wild type full length M segment did not. Like in the case with the RdRp gene sequence, the GP gene sequence of TSWV was also predicted to contain numerous intron splicing sites (*SI Appendix, Table S2*). The absence of antigenomic and genomic RNA strands from the wild type full length M replicon on Northern blots (Fig. S7B) indicated the

possibility that primary transcripts could have been prone to splicing in the GP sequence. This would not only lead to a loss of genome length RNA molecules, but also inhibit the production of NSm protein (either from direct translation of the primary M transcript, or after secondary transcription of NSm mRNA), needed for cell-to-cell and systemic movement of viral RNPs.

Not only the wild type sequence of L and M RNA segments may be spliced in the nucleus, also the S RNA segment generated by the 35S promoter could be prone to splicing. This is supported by the experiments in which *N. benthamiana* where infiltrated with the S<sub>(+)</sub>eGFP mini-replicon, M<sub>(-)</sub>opt and L<sub>(+)</sub>opt and resulted in 80% virus recovery (Table 1), but when only the S<sub>(+)</sub>eGFP mini-replicon was exchanged for the full length S<sub>(+)</sub> virus recovery dropped to 11.37 %. The very same reason may explain the low infection rate (10 %) when full length S<sub>(+)</sub>, M<sub>(-)</sub>opt and L<sub>(+)</sub>opt are co-expressed (Table 1). Although this could be due to the splicing of S RNA, the (residual) levels of full length S produced apparently have been sufficient to initiate viral replication.

Similar to other bunyaviruses (39, 52, 57), both the RdRp and N proteins are required for reconstitution of infectious RNPs complexes for TSWV (Fig 1C). However, high expression of either N or RdRp results in cell death and cause negative effects on the replication and transcription of TSWV. Moreover, ectopic expression of N and RdRp by the strong 35S promoter also seems to cause superinfection exclusion, as earlier observed and reported with various viruses infecting humans, animals, and plants (47, 58-60). During superinfection exclusion a preexisting infection of virus prevents a secondary infection with the same or a highly similar virus. It is an active

virus-controlled process that may be determined by a specific viral protein. For example, for potyvirus the coat protein and NIa protease have been identified to control superinfection exclusion (60). For TCV, the p28, involved in replication protein, was shown to confer superinfection exclusion as *a priori* expression of p28 blocked (re-)infection with TCV (47). Ectopic expression of N and RdRp may also have blocked progeny L-, M- and S- RNAs from moving into neighboring plant cells. However, the presence of L-, M- and S- RNA segments in the same cells is a pre-requisite for the reconstitution of infectious TSWV and to systemic spread in *N. benthamiana*. Fortunately, direct expression of N from S<sub>(+)</sub> and RdRp from L<sub>(+)</sub>opt have helped to overcome superinfection exclusion and to recover infectious TSWV. Whether this is due to the fact that N and RdRp are directly expressed from primary viral genome transcripts and simultaneously associate to progeny L-, M- and S- RNA segments in the same plant cells into infectious RNPs and/or involves a more fine-tuned protein expression relative to RNA segment replication remains unclear. Ectopic expression of NSs also inhibited the rescue of full length S<sub>(+)</sub> segment from cDNA. Since TSWV NSs significantly enhanced the replication of S and M mini-replicons lacking the NSs ORF, this indicated that the inhibition could relate to (simultaneous / *a priori*) ectopic expression of NSs gene sequences with overlap to the full length S<sub>(+)</sub> replicon. This could be tested by ectopic expression of an untranslatable NSs<sup>ΔATG</sup> construct.

In summary, a series of issues has hampered the construction of a successful TSWV reverse genetics system: the choice of promoter and construct design to



generate primary viral RNA transcripts in plants that mimick authentic viral RNA molecules, the expression of a very large viral RdRp, negative effects of ectopic expression of RdRp, N and NSs, and the absence of viral RNA synthesis of the wild type M<sub>(-)</sub> segment. In this study, we have been able to solve all these issues and successfully managed to establish a reverse genetics system for the tripartite RNA genome of TSWV. Using the S RNA mini-replicon system containing eGFP and mCherry reporter genes, the role of *cis*- and *trans*-acting elements for viral replication and transcription can be studied. Using the M-RNA mini-replicon system cell-to-cell movement of TSWV RNPs *in planta* can be studied. To track the virus during systemic infection of plants rTSWV can be generated containing fluorescent reporter genes at the genetic loci of either GP or NSs. The establishment of this RG system now provides us with a new and powerful platform to generate mutant viruses and study their disease pathology in a natural setting, including basic principles of all tospovirus life cycle and viral pathogenicity. As a personal communication, Jeanmarie Verchot's group has also recovered the Rose rosette emaravirus entirely from cDNA clones, a plant NSV with 7 RNA segments. The establishment of these RG systems presents the start of a new research era for the segmented plant NSVs.

## Materials and Methods

Details of the methodology used are provided in [SI Appendix, Materials and Methods](#), and include plasmid construction, plant material and growth conditions, agro-infiltration, immunoblot analysis, Northern blot analysis, RT-PCR, GFP imaging,

fluorescence microscopy and Electron microscopy. Primers used in this study are listed in [SI Appendix, Table S3](#).

## ACKNOWLEDGMENTS

We thank Dr. Yi Xu for critical review of this manuscript. This work was supported by the National Natural Science Foundation of China (31630062 and 31471746), the Fundamental Research Funds for the Central Universities (JCQY201904), Youth Science and Technology Innovation Program to XT and Postgraduate Research & Practice Innovation Program of Jiangsu Province to MF.

## References

1. Fields BN, Knipe, DM, & Howley, PM (1996) *Fields Virology* (Lippincott-Raven, New York).
2. Elliott RM, Blakqori G (2011) Molecular biology of orthobunyaviruses. In: Plyusnin, A, Elliott, RM (Eds.), *The Bunyaviridae: molecular and cellular biology*. *Horizon Scientific Press*, Norwhich, UK.
3. German TL, Ullman DE, & Moyer JW (1992) Tospoviruses: diagnosis, molecular biology, phylogeny, and vector relationships. *Annu Rev Phytopathol* 30:315-348.
4. Kong L, Wu J, Lu L, Xu Y, & Zhou X (2014) Interaction between rice stripe virus disease-specific protein and host PsbP enhances virus symptoms. *Mol Plant* 7(4):691-708.
5. Lu G, *et al.* (2019) Tenuivirus utilizes its glycoprotein as a helper component to overcome insect midgut barriers for its circulative and propagative transmission. *PLoS Pathog* 15(3):e1007655.
6. Oliver JE & Whitfield AE (2016) The genus tospovirus: emerging bunyaviruses that threaten food security. *Annu Rev Virol* 3(1):101-124.
7. Prins M & Goldbach R (1998) The emerging problem of tospovirus infection and nonconventional methods of control. *Trends Microbiol* 6(1):31-35.
8. Turina M, Kormelink R, & Resende RO (2016) Resistance to tospoviruses in vegetable crops: epidemiological and molecular aspects. *Annu Rev Phytopathol* 54:347-371.
9. Whitfield AE, Ullman DE, & German TL (2005) Tospovirus-thrips interactions. *Annu Rev Phytopathol* 43:459-489.
10. Zhu M, van Grinsven IL, Kormelink R, & Tao X (2019) Paving the way to tospovirus infection: multilined interplays with plant innate immunity. *Annu Rev Phytopathol* 57:2.1-2.22.

- 584 11. Kormelink R, Garcia ML, Goodin M, Sasaya T, & Haenni AL (2011) Negative-strand RNA  
585 viruses: the plant-infecting counterparts. *Virus Res* 162(1-2):184-202.
- 586 12. Scholthof KB, *et al.* (2011) Top 10 plant viruses in molecular plant pathology. *Mol Plant*  
587 *Pathol* 12(9):938-954.
- 588 13. Adams MJ, *et al.* (2017) Changes to taxonomy and the international code of virus  
589 classification and nomenclature ratified by the international committee on taxonomy of  
590 viruses. *Arch Virol* 162(8):2505-2538.
- 591 14. Pappu HR, Jones RA, & Jain RK (2009) Global status of tospovirus epidemics in diverse  
592 cropping systems: successes achieved and challenges ahead. *Virus Res* 141(2):219-236.
- 593 15. Hogenhout SA, Ammar el D, Whitfield AE, & Redinbaugh MG (2008) Insect vector  
594 interactions with persistently transmitted viruses. *Annu Rev Phytopathol* 46:327-359.
- 595 16. Gilbertson RL, Batuman O, Webster CG, & Adkins S (2015) Role of the insect superectors  
596 Bemisia tabaci and Frankliniella occidentalis in the emergence and global spread of plant  
597 viruses. *Annu Rev Virol* 2(1):67-93.
- 598 17. Adkins S, Quadt R, Choi TJ, Ahlquist P, & German T (1995) An RNA-dependent RNA  
599 polymerase activity associated with virions of tomato spotted wilt virus, a plant- and  
600 insect-infecting bunyavirus. *Virology* 207(1):308-311.
- 601 18. de Haan P, *et al.* (1991) Tomato spotted wilt virus L RNA encodes a putative RNA polymerase.  
602 *J Gen Virol* 72 ( Pt 9):2207-2216.
- 603 19. Kikkert M, *et al.* (1999) Tomato spotted wilt virus particle morphogenesis in plant cells. *J*  
604 *Virol* 73(3):2288-2297.
- 605 20. Ribeiro D, *et al.* (2008) Tomato spotted wilt virus glycoproteins induce the formation of  
606 endoplasmic reticulum- and Golgi-derived pleomorphic membrane structures in plant cells. *J*  
607 *Gen Virol* 89(Pt 8):1811-1818.
- 608 21. Sin SH, McNulty BC, Kennedy GG, & Moyer JW (2005) Viral genetic determinants for thrips  
609 transmission of tomato spotted wilt virus. *Proc Natl Acad Sci U S A* 102(14):5168-5173.
- 610 22. Feng Z, *et al.* (2016) The ER-membrane transport system is critical for intercellular trafficking  
611 of the NSm movement protein and tomato spotted wilt tospovirus. *PLoS Pathog*  
612 12(2):e1005443.
- 613 23. Kormelink R, Storms M, Van Lent J, Peters D, & Goldbach R (1994) Expression and  
614 subcellular location of the NSm protein of tomato spotted wilt virus (TSWV), a putative viral  
615 movement protein. *Virology* 200(1):56-65.
- 616 24. Soellick T, Uhrig JF, Bucher GL, Kellmann JW, & Schreier PH (2000) The movement protein  
617 NSm of tomato spotted wilt tospovirus (TSWV): RNA binding, interaction with the TSWV N  
618 protein, and identification of interacting plant proteins. *Proc Natl Acad Sci U S A*  
619 97(5):2373-2378.
- 620 25. Storms MM, Kormelink R, Peters D, Van Lent JW, & Goldbach RW (1995) The nonstructural  
621 NSm protein of tomato spotted wilt virus induces tubular structures in plant and insect cells.  
622 *Virology* 214(2):485-493.
- 623 26. Storms MM, *et al.* (1998) A comparison of two methods of microinjection for assessing  
624 altered plasmodesmal gating in tissues expressing viral movement proteins. *Plant J*  
625 13(1):131-140.
- 626 27. Li J, *et al.* (2015) Structure and function analysis of nucleocapsid protein of tomato spotted  
627 wilt virus interacting with RNA using homology modeling. *J Biol Chem* 290(7):3950-3961.

- 628 28. Komoda K, Narita M, Yamashita K, Tanaka I, & Yao M (2017) Asymmetric trimeric ring  
629 structure of the nucleocapsid protein of tospovirus. *J Virol* 91(20):e01002-17.
- 630 29. Guo Y, *et al.* (2017) Distinct mechanism for the formation of the ribonucleoprotein complex  
631 of tomato spotted wilt virus. *J Virol* 91(23):e00892-17.
- 632 30. Feng Z, *et al.* (2013) Nucleocapsid of tomato spotted wilt tospovirus forms mobile particles  
633 that traffic on an actin/endoplasmic reticulum network driven by myosin XI-K. *New Phytol*  
634 200(4):1212-1224.
- 635 31. Ribeiro D, *et al.* (2013) The cytosolic nucleoprotein of the plant-infecting bunyavirus tomato  
636 spotted wilt recruits endoplasmic reticulum-resident proteins to endoplasmic reticulum export  
637 sites. *Plant Cell* 25(9):3602-3614.
- 638 32. Bucher E, Sijen T, De Haan P, Goldbach R, & Prins M (2003) Negative-strand tospoviruses  
639 and tenuiviruses carry a gene for a suppressor of gene silencing at analogous genomic  
640 positions. *J Virol* 77(2):1329-1336.
- 641 33. Schnettler E, *et al.* (2010) Diverging affinity of tospovirus RNA silencing suppressor proteins,  
642 NSs, for various RNA duplex molecules. *J Virol* 84(21):11542-11554.
- 643 34. Takeda A, *et al.* (2002) Identification of a novel RNA silencing suppressor, NSs protein of  
644 tomato spotted wilt virus. *FEBS Lett* 532(1-2):75-79.
- 645 35. Hoang NH, Yang HB, & Kang BC (2013) Identification and inheritance of a new source of  
646 resistance against tomato spotted wilt virus (TSWV) in Capsicum. *Sci Horti-Amsterdam*  
647 161:8-14.
- 648 36. de Ronde D, *et al.* (2014) Analysis of tomato spotted wilt virus NSs protein indicates the  
649 importance of the N-terminal domain for avirulence and RNA silencing suppression. *Mol*  
650 *Plant Pathol* 15(2):185-195.
- 651 37. Kim SB, *et al.* (2017) Divergent evolution of multiple virus-resistance genes from a  
652 progenitor in Capsicum spp. *New Phytol* 213(2):886-899.
- 653 38. Brittlebank CC (1919) Tomato diseases. *J Agri Victoria* 27:231-235.
- 654 39. Bridgen A & Elliott RM (1996) Rescue of a segmented negative-strand RNA virus entirely  
655 from cloned complementary DNAs. *Proc Natl Acad Sci U S A* 93(26):15400-15404.
- 656 40. Neumann G, *et al.* (1999) Generation of influenza A viruses entirely from cloned cDNAs.  
657 *Proc Natl Acad Sci U S A* 96(16):9345-9350.
- 658 41. Flatz L, Bergthaler A, de la Torre JC, & Pinschewer DD (2006) Recovery of an arenavirus  
659 entirely from RNA polymerase I/II-driven cDNA. *Proc Natl Acad Sci U S A*  
660 103(12):4663-4668.
- 661 42. Ganesan U, *et al.* (2013) Construction of a sonchus yellow net virus mini-replicon: a step  
662 toward reverse genetic analysis of plant negative-strand RNA viruses. *J Virol*  
663 87(19):10598-10611.
- 664 43. Wang Q, *et al.* (2015) Rescue of a plant negative-strand RNA virus from cloned cDNA:  
665 insights into enveloped plant virus movement and morphogenesis. *PLoS Pathog*  
666 11(10):e1005223.
- 667 44. Ishibashi K, Matsumoto-Yokoyama E, & Ishikawa M (2017) A tomato spotted wilt virus S  
668 RNA-based replicon system in yeast. *Sci Rep* 7(1):12647.
- 669 45. van Knippenberg I, Goldbach R, & Kormelink R (2005) Tomato spotted wilt virus S-segment  
670 mRNAs have overlapping 3'-ends containing a predicted stem-loop structure and conserved  
671 sequence motif. *Virus Res* 110(1-2):125-131.

- 672 46. de Haan P, Wagemakers L, Peters D, & Goldbach R (1990) The S RNA segment of tomato  
673 spotted wilt virus has an ambisense character. *J Gen Virol* 71 ( Pt 5):1001-1007.
- 674 47. Zhang XF, *et al.* (2017) A self-perpetuating repressive state of a viral replication protein  
675 blocks superinfection by the same virus. *PLoS Pathog* 13(3):e1006253.
- 676 48. Elliott RM (2014) Orthobunyaviruses: recent genetic and structural insights. *Nat Rev*  
677 *Microbiol* 12(10):673-685.
- 678 49. Ferron F, Weber F, de la Torre JC, & Reguera J (2017) Transcription and replication  
679 mechanisms of bunyaviridae and arenaviridae L proteins. *Virus Res* 234:118-134.
- 680 50. Blakqori G & Weber F (2005) Efficient cDNA-based rescue of La Crosse bunyaviruses  
681 expressing or lacking the nonstructural protein NSs. *J Virol* 79(16):10420-10428.
- 682 51. Ikegami T, Won S, Peters CJ, & Makino S (2006) Rescue of infectious rift valley fever virus  
683 entirely from cDNA, analysis of virus lacking the NSs gene, and expression of a foreign gene.  
684 *J Virol* 80(6):2933-2940.
- 685 52. Flick R & Pettersson RF (2001) Reverse genetics system for uukuniemi virus (Bunyaviridae):  
686 RNA polymerase I-catalyzed expression of chimeric viral RNAs. *J Virol* 75(4):1643-1655.
- 687 53. Hempel WM, Cavanaugh AH, Hannan RD, Taylor L, & Rothblum LI (1996) The  
688 spezhucies-specific RNA polymerase I transcription factor SL-1 binds to upstream binding  
689 factor. *Mol Cell Biol* 16(2):557-563.
- 690 54. Doelling JH, Gaudino RJ, & Pikaard CS (1993) Functional analysis of Arabidopsis thaliana  
691 rRNA gene and spacer promoters in vivo and by transient expression. *Proc Natl Acad Sci U S*  
692 *A* 90(16):7528-7532.
- 693 55. SaezVasquez J & Pikaard CS (1997) Extensive purification of a putative RNA polymerase I  
694 holoenzyme from plants that accurately initiates rRNA gene transcription in vitro. *P Natl Acad*  
695 *Sci USA* 94(22):11869-11874.
- 696 56. Martin A, Staeheli P, & Schneider U (2006) RNA polymerase II-controlled expression of  
697 antigenomic RNA enhances the rescue efficacies of two different members of the  
698 Mononegavirales independently of the site of viral genome replication. *J Virol*  
699 80(12):5708-5715.
- 700 57. Flick R, Flick K, Feldmann H, & Elgh F (2003) Reverse genetics for crimean-congo  
701 hemorrhagic fever virus. *J Virol* 77(10):5997-6006.
- 702 58. Laliberte JP & Moss B (2014) A novel mode of poxvirus superinfection exclusion that  
703 prevents fusion of the lipid bilayers of viral and cellular membranes. *J Virol*  
704 88(17):9751-9768.
- 705 59. Webster B, Ott M, & Greene WC (2013) Evasion of superinfection exclusion and elimination  
706 of primary viral RNA by an adapted strain of hepatitis C virus. *J Virol* 87(24):13354-13369.
- 707 60. Tatineni S & French R (2016) The coat protein and NIa protease of two potyviridae family  
708 members independently confer superinfection exclusion. *J Virol* 90(23):10886-10905.

709  
710  
711  
712  
713

**Table 1.** Systemic infection rate of recombinant TSWV rescued in *N. benthamiana* in the presence of viral suppressors of RNA silencing (VSRs).

Antigenome and genome derivatives	VSRs	Systemic infection
		(No. of infected/inoculated plants)
S <sub>(-)</sub> +M <sub>(-)</sub> +L <sub>(-)</sub>	N+RdRp+NSs+P19-HcPro-γb	0 % (0/30)
S <sub>(+)</sub> +M <sub>(+)</sub> +L <sub>(+)</sub>	N+RdRp+NSs+P19-HcPro-γb	0 % (0/30)
SM <sub>(+)</sub> eGFP+MR <sub>(-)</sub> mCherry+L <sub>(+)</sub> opt	NSs+P19-HcPro-γb	80 % (24/30)
S <sub>(+)</sub> +MR <sub>(-)</sub> eGFP+L <sub>(+)</sub> opt	P19-HcPro-γb	11.37 % (7/60)
S <sub>(+)</sub> +MR <sub>(-)</sub> eGFP+L <sub>(+)</sub> opt	NSs+P19-HcPro-γb	0 % (0/60)
SR <sub>(+)</sub> eGFP+M <sub>(-)</sub> +L <sub>(+)</sub> opt	P19-HcPro-γb	0 % (0/60)
SR <sub>(+)</sub> eGFP+M <sub>(-)</sub> +L <sub>(+)</sub> opt	NSs+P19-HcPro-γb	0 % (0/60)
SR <sub>(+)</sub> eGFP+M <sub>(-)</sub> opt+L <sub>(+)</sub> opt	NSs+P19-HcPro-γb	90 % (27/30)
S <sub>(+)</sub> +M <sub>(-)</sub> opt+L <sub>(+)</sub> opt	P19-HcPro-γb	10 % (6/60)

716

717 Mixture of *Agrobacterium* cultures harboring the plasmids encoding each of the S, M, L and  
 718 derivatives (final concentration OD<sub>600</sub>=0.2), RdRp (OD<sub>600</sub>=0.2), N (OD<sub>600</sub>=0.2) and VSRs  
 719 (OD<sub>600</sub>=0.05) were infiltrated into *N. benthamiana* leaves. Systemic infection was scored  
 720 15-30 dpi.

721

722

723

724

725

726

727

## Figure Legends:

**Fig. 1** Construction of a TSWV S<sub>(-)</sub> RNA-based mini-replicon system in *N. benthamiana*. (A) Schematic representation of binary constructs to express TSWV S<sub>(-)</sub> mini-replicon, TSWV N, RdRp and four RNA silencing suppressors (VSRs: NSs, P19, HcPro and γb) proteins by agroinfiltration into *N. benthamiana*. The S<sub>(-)</sub>-gRNA of TSWV is shown on the top. SR<sub>(-)</sub>mCherry&eGFP: the NSs and N of S<sub>(-)</sub>-gRNA were replaced by mCherry and eGFP, respectively. (-) refers to the negative (genomic)-strand of S RNA; 2×35S: a double 35S promoter; HH: hammerhead ribozyme; RZ: Hepatitis Delta virus (HDV) ribozyme; NOS: nopaline synthase terminator. (B) Foci of eGFP and mCherry fluorescence in *N. benthamiana* leaves co-expressing SR<sub>(-)</sub>mCherry&eGFP, RdRp, N and four VSRs at 5 days post infiltration (dpi) under a fluorescence microscope. The bar represents 400 μm. (C) Analysis of RdRp and N requirement for SR<sub>(-)</sub>mCherry&eGFP mini-genome replication in *N. benthamiana* leaves. SR<sub>(-)</sub>mCherry&eGFP was coexpressed with pCB301 empty vector (Vec), N, RdRp or both in *N. benthamiana* leaves by agroinfiltration. Agro-infiltrated leaves were examined and photographed at 5 dpi under a fluorescence microscope. Signal shown reflects a merge of mCherry and eGFP fluorescence from both reporter genes. Bar represents 400 μm. (D) Immunoblot analysis on the expression of N and eGFP proteins in the leaves shown in panel (C) using specific antibodies against N and GFP, respectively. Ponceau S staining of rubisco large subunit is shown for protein loading control. (E) Northern blot analysis of S<sub>(-)</sub>-mini-replicon replication and transcription in the presence of N, RdRp or both in *N. benthamiana*. The S RNA genomic,



anti-genomic and subgenomic transcripts (eGFP mRNA) were detected by DIG-labeled sense eGFP or anti-sense eGFP probes. The red and blue arrows indicate the anti-genomic and genomic RNAs of SR<sub>(-)</sub>mCherry&eGFP, respectively. The green arrow indicates the eGFP mRNA transcript. Ethidium bromide staining of ribosomal RNA (rRNA) was used as RNA loading control.

**Fig. 2** Optimization of the SR<sub>(-)</sub>mCherry&eGFP mini-replicon system. (A) Optimizing the concentration of N and RdRp proteins for replication and transcription of SR<sub>(-)</sub>mCherry&eGFP in *N. benthamiana* leaves. Increasing amounts of *Agrobacterium*, from OD<sub>600</sub>= 0.2 to 0.8 and containing the binary expression constructs for N (upper panels) or RdRp (bottom panels), were mixed with fixed amounts of *Agrobacterium* containing the RdRp or N construct (OD<sub>600</sub> 0.2), respectively, and their effects on eGFP reporter expression were visualized under a fluorescence microscope at 5 dpi. Bars represent 400 μm. (B) and (C) Western immunoblot detection of the N and eGFP proteins expressed in the leaves shown in panel (A) using specific antibodies against N and GFP, respectively. (D) Optimization of RNA silencing suppressors (VSRs) on SR<sub>(-)</sub>mCherry&eGFP mini-reporter replication and transcriptions as measured by eGFP and mCherry expression. The SR<sub>(-)</sub>mCherry&eGFP, N and RdRp proteins were co-expressed with pCB301 empty vector (Vec), NSs, P19-HcPro-γb or all four VSRs in *N. benthamiana* leaves. Foci expressing eGFP and mCherry in agroinfiltrated leaves were visualized under a fluorescence microscope at 5 dpi. Bars represent 400 μm. (E) Western immunoblot detection of N and eGFP protein synthesis in the leaves shown



in panel (D) using N and GFP-specific antibodies, respectively. Ponceau S staining was used as protein loading control.

**Fig. 3** Development of the S<sub>(+)</sub>-agRNA mini-replicon system in *N. benthamiana*. (A) Schematic representation of the TSWV SR<sub>(+)</sub>eGFP mini-replicon. The NSs gene of TSWV S agRNA was replaced by eGFP. Anti-genomic RNA strands of the SR<sub>(+)</sub>eGFP mini-replicon are transcribed from a double 35S promoter (2×35S), and flanked by a HH ribozyme and HDV ribozyme (RZ) sequence. (+) refers to the positive (antigenomic)-strand of S RNA. (B) Foci of eGFP fluorescence in *N. benthamiana* leaves co-expressing TSWV SR<sub>(+)</sub>eGFP with pCB301 empty vector (Vec), N, RdRp or N+RdRp by agroinfiltration. The agroinfiltrated leaves were photographed at 3 dpi under a fluorescence microscope. Bars represent 400 μm. (C) Western immunoblot detection of N and eGFP protein synthesis in the leaves shown in panel (B) using N- and GFP-specific antibodies, respectively. Ponceau S staining was used as a protein loading control. (D) Northern blot analysis of the replication and transcription of SR<sub>(+)</sub>eGFP mini-replicon in *N. benthamiana* co-expressed with empty vector (Vec), N, RdRp or both. The anti-genomic RNAs (red arrow), genomic RNAs (blue arrow) and eGFP mRNA transcripts (green arrow) were detected with DIG-labeled sense and anti-sense eGFP probes, respectively. Ethidium bromide staining was used as RNA loading control.

**Fig. 4** Establishment of a TSWV M<sub>(-)</sub>-gRNA based mini-replicon system with

cell-to-cell movement competency in *N. benthamiana*. (A) Schematic representation of the TSWV MR<sub>(-)</sub>eGFP mini-replicon and its mutant derivative MR<sub>(-)</sub>eGFP&NSmMut. The GP gene of TSWV M<sub>(-)</sub> gRNA was substituted by eGFP. The genomic RNA of the mini-replicon is transcribed from a double 35S promoter (2×35S) and flanked by a Hammerhead (HH) ribozyme and HDV ribozyme (RZ). For MR<sub>(-)</sub>eGFP&NSmMut, a stop codon was introduced immediately after the start codon of the NSm gene in the MR<sub>(-)</sub>eGFP mini-replicon. (B) Foci of eGFP fluorescence expressed from the MR<sub>(-)</sub>eGFP or MR<sub>(-)</sub>eGFP&NSmMut mini-replicon in *N. benthamiana* leaves co-expressed with the empty vector (Vec), N, RdRp or N+RdRp by agroinfiltration. Agroinfiltrated leaves were photographed at 4 dpi under a fluorescence microscope. Bars represent 400 μm. (C) Western immunoblot detection of N and eGFP protein synthesis in the leaves shown in panel (B) with specific antibodies against N and GFP, respectively. Ponceau S staining was used as protein loading control. (D) Northern blot analysis of the replication and transcription of the MR<sub>(-)</sub>eGFP mini-replicon co-expressed with Vec, N, RdRp or N+RdRp in *N. benthamiana* leaves. The anti-genomic RNAs (red arrow), genomic RNAs (blue arrow) and eGFP mRNA transcripts (green arrow) were detected with DIG labeled sense and anti-sense eGFP probes, respectively. Ethidium bromide staining was used as RNA loading control.

**Fig. 5** Establishment of a systemic infection in *N. benthamiana* with replicons S<sub>(+)</sub> and M<sub>(-)</sub> co-expressed with full length antigenomic L<sub>(+)</sub>opt. (A) Schematic representation of constructs expressing TSWV full length antigenomic L<sub>(+)</sub>opt with optimized RdRp, full

length genomic  $M_{(-)opt}$  with optimized GP,  $MR_{(-)eGFP}$ ,  $MR_{(-)mCherry}$ , full length antigenomic  $S_{(+)}$  and  $SR_{(+)eGFP}$ . Primary viral RNA transcripts are transcribed from a double 35S promoter ( $2 \times 35S$ ) and flanked by a HH and HDV ribozyme (RZ). (B) eGFP and mCherry fluorescence in *N. benthamiana* resulting from systemic infection of agroinfiltrated  $SR_{(+)eGFP}$ ,  $MR_{(-)mCherry}$  and  $L_{(+)opt}$  constructs. The systemic infected plants (a and b) and leaves (c and d) were photographed at 21 dpi under white light and (hand-held) ultraviolet (UV) light. Foci of eGFP and mCherry fluorescence in leaves shown in panel d as visualized under a fluorescence microscope. Bar represents 400  $\mu m$ . (C) eGFP fluorescence in *N. benthamiana* resulting from systemic infection of agroinfiltrated  $SR_{(+)eGFP}$ ,  $M_{(-)opt}$  and  $L_{(+)opt}$  constructs. Infected plants (a and b) and leaves (c and d) were photographed at 18 dpi under white light and (hand-held) UV light, respectively. (D) eGFP fluorescence in *N. benthamiana* resulting from systemic infection of agroinfiltrated  $S_{(+)}$ ,  $MR_{(-)eGFP}$  and  $L_{(+)opt}$  constructs. Infected plants (a and b) and leaves (c and d) were photographed at 50 dpi under white light and (hand-held) UV light, respectively.

**Fig. 6** Rescue of infectious TSWV from full-length cDNA clones in *N. benthamiana*.

(A) Systemic infection of *N. benthamiana* plants with rescued TSWV (rTSWV) resulting from agroinfiltration of  $S_{(+)}$ ,  $M_{(-)opt}$  and  $L_{(+)opt}$  and three VSRs (P19, HcPro and  $\gamma b$ ). The plant agroinfiltrated with pCB301 empty vector was used as a mock control. Images were taken at 19 dpi. Boxed areas of the plants that show stunting, mosaic and leaf curling are shown enlarged in the right panels. (B) Sequence

838 confirmation of codon optimized sequences of the GP gene (from the M<sub>(-)opt</sub> RNA  
839 segment) and the RdRp gene (from the L<sub>(+)opt</sub> RNA segment) on RT-PCR fragments  
840 obtained from systemic leaves of *N. benthamiana* infected with rTSWV. The  
841 optimized sequence of GP from rTSWV is underlined by a blue dashed line, and wild  
842 type GP sequence underlined in blue. The 3'-untranslated region (UTR) sequence of  
843 the M genomic RNA is marked with a yellow line. The optimized sequence of RdRp  
844 from rTSWV is underlined by a red dashed line, and wild type RdRp sequence  
845 underlined in red. The 3'-UTR sequence of the L genomic RNA is marked with a  
846 purple line. The stars indicate the codon optimization sites of GP and RdRp gene  
847 sequences. (C) Northern blot detection of viral RNA from the S, M and L RNA  
848 segment, respectively, in systemic leaves of *N. benthamiana* infected with rTSWV.  
849 The anti-genomic RNAs (red arrow) and genomic RNAs (blue arrow) were detected  
850 with DIG labeled sense and anti-sense NSs-, NSm- and L-5'UTR probes, respectively.  
851 Lane 1 and 2 refer to two independent replicates. Ethidium bromide staining was used  
852 as RNA loading control. (D) Western immunoblot detection of the N, NSm, NSs, Gc,  
853 and Gn proteins from leaves systemically infected with rTSWV, using specific  
854 antibodies against N, NSm, NSs, Gc, and Gn, respectively. Leaves infected with  
855 wild-type TSWV were used as a positive control. Ponceau S staining was used as  
856 protein loading control. (E) Electron micrographs of thin sections of *N. benthamiana*  
857 plants infected with rTSWV. Boxed regions in the left panels and showing the  
858 presence of virions, are shown enlarged in the right panels. Spherical enveloped virus  
859 particles are indicated (white arrow head). Bars represent 0.2  $\mu$ m.

## Supplemental Figure Legends

**Fig. S1** Functional analysis of wild type RdRp and the use of T7 promoter in a mini-genome replication assay. (A) Schematic diagram of TSWV 35S:SR<sub>(-)</sub>mCherry&eGFP and T7:SR<sub>(-)</sub>mCherry&eGFP mini-replicon reporters. (B) The wild type RdRp (RdRp<sub>wt</sub>) or the empty vector (Vec) was co-expressed with 35S:SR<sub>(-)</sub>mCherry&eGFP, N, VSRs (VSRs: NSs, P19, HcPro and  $\gamma$ b) in *N. benthamiana* leaves. The expression of eGFP was examined by a fluorescence microscope. (C) Constructs coding for T7:SR<sub>(-)</sub>mCherry&eGFP, T7 RNA polymerase (pol), N and VSRs were co-expressed with RdRp<sub>wt</sub> or RdRp<sub>opt</sub> in *N. benthamiana* leaves. Replication of T7:SR<sub>(-)</sub>mCherry&eGFP was examined by monitoring for eGFP fluorescence with a fluorescence microscope. Empty vector (Vec) pCB301 was used as a negative control. Bars represent 200  $\mu$ m. (D) Western immunoblot detection of T7 RNA pol using a T7 RNA pol specific antibody. Ponceau S staining was used as protein loading control. Lane 1: sample from leaves co-expressing T7:SR<sub>(-)</sub>mCherry&eGFP, T7 RNA Pol, N, VSRs and RdRp<sub>wt</sub>; lane 2: sample from leaves co-expressing T7:SR<sub>(-)</sub>mCherry&eGFP, T7 RNA Pol, N, VSRs and RdRp<sub>opt</sub>.

**Fig. S2** Time course analysis on gene expression from the SR<sub>(-)</sub>mCherry&eGFP mini-replicon in *N. benthamiana* leaves. (A) Foci of eGFP and mCherry fluorescence expressed from SR<sub>(-)</sub>mCherry&eGFP in *N. benthamiana* leaves co-expressing N, RdRp and the VSRs at 3, 6, 9 and 12 dpi, respectively. Fluorescence of eGFP and mCherry were photographed under a fluorescence microscope using GFP and RFP filters,

respectively. Bars represent 400  $\mu$ m. (B) Western immunoblot detection of the N, eGFP and mCherry proteins in leaves shown in panel A, using specific antibodies against N, GFP and mCherry, respectively. The empty vector (Vec) was used as a negative control. Ponceau S staining was used as protein loading control.

**Fig. S3** The role of 5'-UTR, 3'-UTR and IGR on viral RNA synthesis from the SR<sub>(-)</sub>mCherry&eGFP mini-replicon. (A) Schematic representation of TSWV SR<sub>(-)</sub>mCherry&eGFP and derivatives with deletions of the 5'UTR, IGR or 3'UTR. (B) eGFP and mCherry fluorescence expressed from TSWV SR<sub>(-)</sub>mCherry&eGFP and mutant derivatives in *N. benthamiana*. The SR<sub>(-)</sub>mCherry&eGFP or its mutants were coexpressed with N, RdRp and the four VSRs in *N. benthamiana* leaves. The agroinfiltrated leaves were examined and photographed at 5 dpi under a fluorescence microscope using GFP and RFP filters, respectively. Bars represent 200  $\mu$ m. (C) Western immunoblot detection of the N and eGFP proteins expressed from the SR<sub>(-)</sub>mCherry&eGFP mini-replicon and mutant derivatives, using specific antibodies against N and GFP, respectively. Ponceau S staining was used as protein loading control. (D) Northern blot analysis of viral RNA synthesis from SR<sub>(-)</sub>mCherry&eGFP and mutant derivatives. The anti-genomic RNAs (red arrow), genomic RNAs (blue arrow) and eGFP mRNA transcripts (green arrow) were detected with DIG-labeled sense eGFP or anti-sense eGFP probes. Ethidium bromide staining was used as RNA loading control.

**Fig. S4** Cell-to-cell movement analysis of SR<sub>(-)</sub>GFP and MR<sub>(-)</sub>mCherry in *N. benthamiana*

co-expressing RdRp, N and four VSRs in *N. benthamiana*. Agroinfiltrated leaves were examined and photographed at 5 dpi under a fluorescence microscope. Bars represent 400  $\mu$ m.

**Fig. S5** Functional analysis of RdRp expressed from TSWV  $L_{(+)\text{opt}}$ , NSm from MR $_{(-)\text{eGFP}}$  and N from SR $_{(+)\text{eGFP}}$  using the mini-genome replication system in *N. benthamiana*. (A) Functional analysis of RdRp expressed from TSWV  $L_{(+)\text{opt}}$  using the S RNA mini-replicon system in *N. benthamiana*. The  $L_{(+)\text{opt}}$ , RdRp, or pCB301 empty vector (Vec) was co-expressed with N, SR $_{(-)\text{mCherry\&eGFP}}$  and the four VSRs in *N. benthamiana* leaves. (B) Functional analysis of N expressed from SR $_{(+)\text{eGFP}}$  in *N. benthamiana*. SR $_{(+)\text{eGFP}}$  was co-expressed with the empty vector (Vec), N, RdRp or N+ $L_{(+)\text{opt}}$  in *N. benthamiana* leaves in the presence of four VSRs. (C) Functional analysis of NSm expressed from MR $_{(-)\text{eGFP}}$  in *N. benthamiana*. MR $_{(-)\text{eGFP}}$  was co-expressed with the empty vector (Vec), N, RdRp or N+ $L_{(+)\text{opt}}$  in *N. benthamiana* leaves in the presence of four VSRs. Foci showing mCherry and eGFP fluorescence in agroinfiltrated *N. benthamiana* leaves were examined at 3 dpi by a fluorescence microscope. Bars represent 400  $\mu$ m.

**Fig. S6** Analysis of *N. benthamiana* leaves agroinfiltrated with constructs of SR $_{(+)\text{eGFP}}$ , MR $_{(-)\text{mCherry}}$  and  $L_{(+)\text{opt}}$ , or SR $_{(+)\text{eGFP}}$ , MR $_{(-)\text{opt}}$  and  $L_{(+)\text{opt}}$  or S $_{(+)}$ , MR $_{(-)\text{eGFP}}$  and  $L_{(+)\text{opt}}$ . (A) Local infection analysis of cell-to-cell movement of SR $_{(+)\text{eGFP}}$  and MR $_{(-)\text{mCherry}}$  co-expressing with  $L_{(+)\text{opt}}$  and four VSRs in *N. benthamiana* by agroinfiltration. The

agro-infiltrated leaves were examined and photographed at 5 dpi under a fluorescence microscope. Bars represent 400  $\mu$ m. (B) RT-PCR analysis on systemically infected leaves from *N. benthamiana* plants agroinfiltrated with SR<sub>(+)</sub>eGFP, MR<sub>(-)</sub>mCherry and L<sub>(+)</sub>opt. (C) RT-PCR analysis on systemically infected leaves from *N. benthamiana* plants agroinfiltrated with S<sub>(+)</sub>, MR<sub>(-)</sub>eGFP and L<sub>(+)</sub>opt. (D) RT-PCR analysis on systemically infected leaves from *N. benthamiana* plants agroinfiltrated with SR<sub>(+)</sub>eGFP, M<sub>(-)</sub>opt and L<sub>(+)</sub>opt. All agroinfiltrations were performed in the additional presence four VSRs constructs. RT PCR was performed on total RNA purified from systemic leaves for detection of S, M or L segments using segment-specific primers. Amplicons were resolved by electrophoresis in a 1 % agarose gel. Lanes 1-2 represent two biological replicates of systemic infected leaf samples; As positive controls (CK<sup>+</sup>) for proper fragment size, PCR was performed on plasmids carrying S, M, L<sub>(+)</sub>opt or derivatives. As negative control (CK<sup>-</sup>), RT-PCR was performed in the absence of nucleic acids. DNA size markers are shown on the left hand side.

**Fig. S7** Northern blot detection of viral RNA synthesis produced from full length S<sub>(+)</sub> and wild type M<sub>(-)</sub> replicons. (A and B) The full length S<sub>(+)</sub> or wild type M<sub>(-)</sub> was co-expressed with the empty vector (Vec), N, RdRp<sub>opt</sub> or N+RdRp<sub>opt</sub> in *N. benthamiana* leaves in the presence of four VSRs. The genomic RNAs (blue arrow), anti-genomic RNAs (red arrow) of S (A) and M (B) were detected with DIG-labeled sense or anti-sense NSs and NSm probes, respectively. Ethidium bromide staining was used as RNA loading control.



948

949 **Fig. S8** RT-PCR detection of S(+), M(-)opt and L(+ )opt genomic RNA in systemic leaves  
 950 of *N. benthamiana* infected by rTSWV. The S(+), M(-)opt and L(+ )opt and the four VSRs  
 951 were co-expressed in *N. benthamiana* leaves by agroinfiltration. Total RNA was  
 952 purified from systemic leaves of agroinfiltrated plants and the presence of S(+), M(-)opt  
 953 and L(+ )opt were detected by RT-PCR using segment-specific primers. RT-PCR  
 954 products were resolved by electrophoresis in a 1% agarose gel. Lanes 1-2, two  
 955 biological replicates of systemic infected leaf samples; RT-PCR on plasmids carrying  
 956 S, M and L as DNA template were used as positive controls (CK<sup>+</sup>). RT-PCR without  
 957 adding the DNA template was used as negative controls (CK<sup>-</sup>). DNA size markers are  
 958 shown on the left hand side.

959

960 **Fig. S9** Optimized RdRp gene sequence used in the study.

961

962 **Fig. S10** Optimized GP gene sequence used in the study.

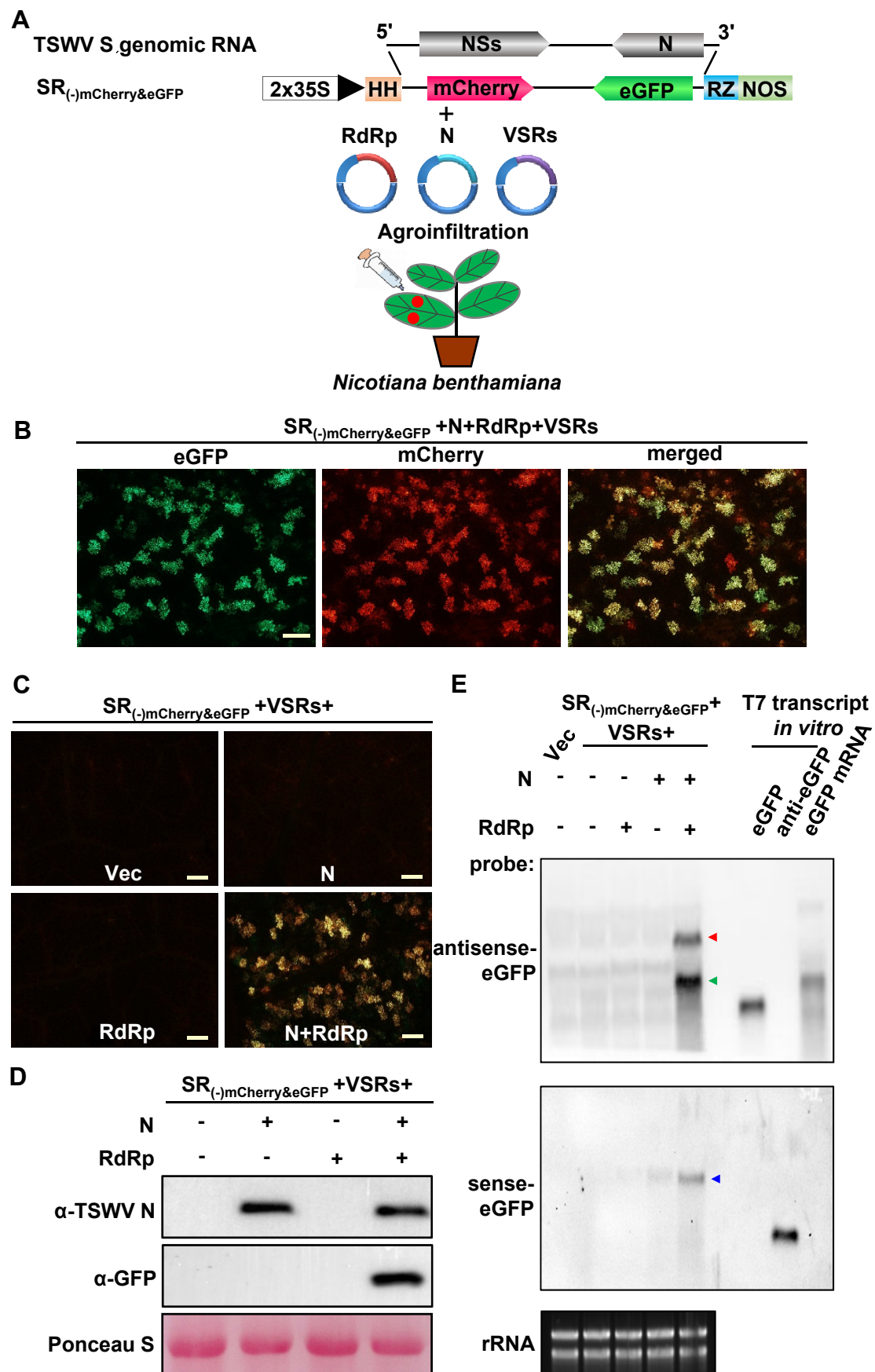
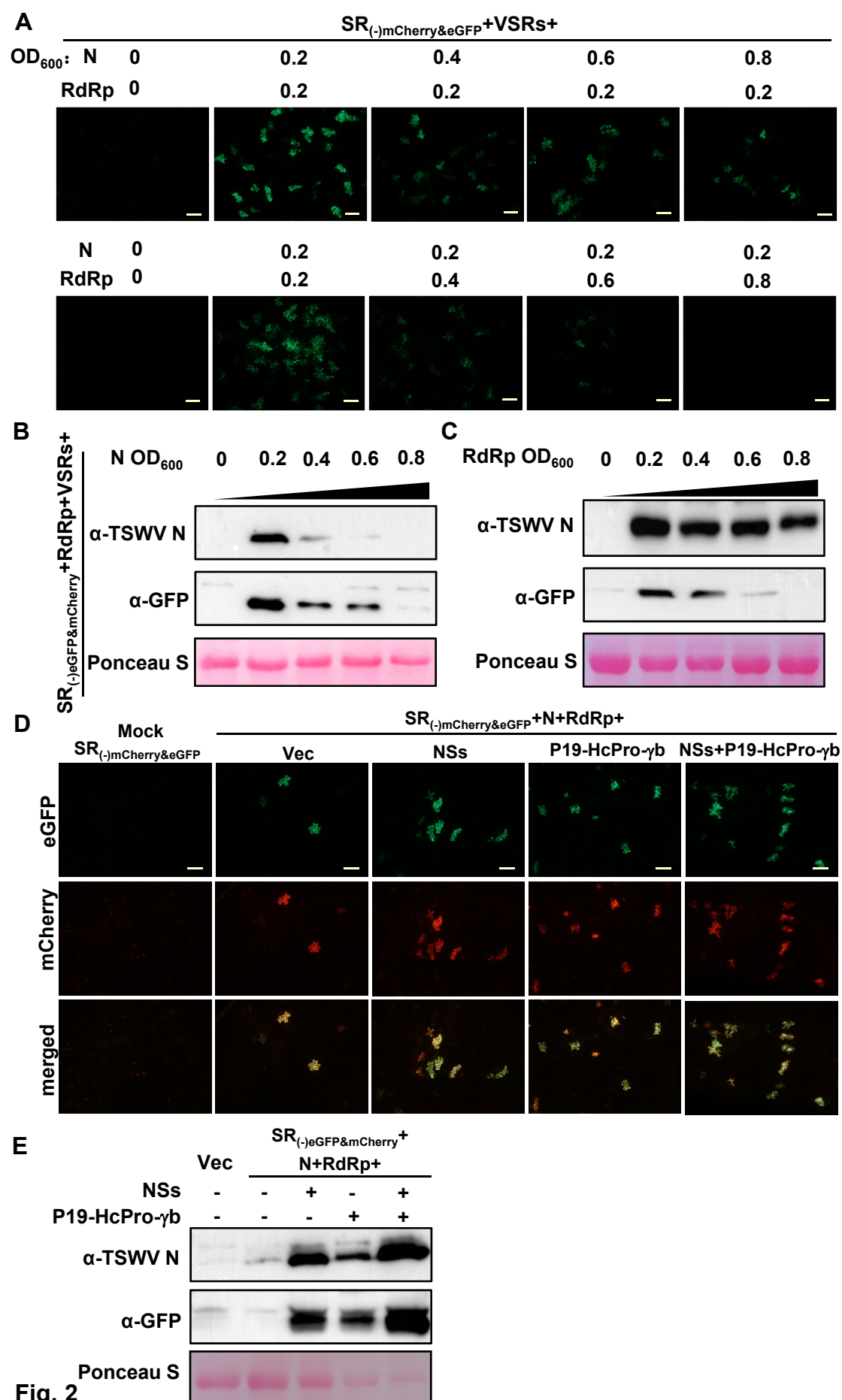


Fig. 1



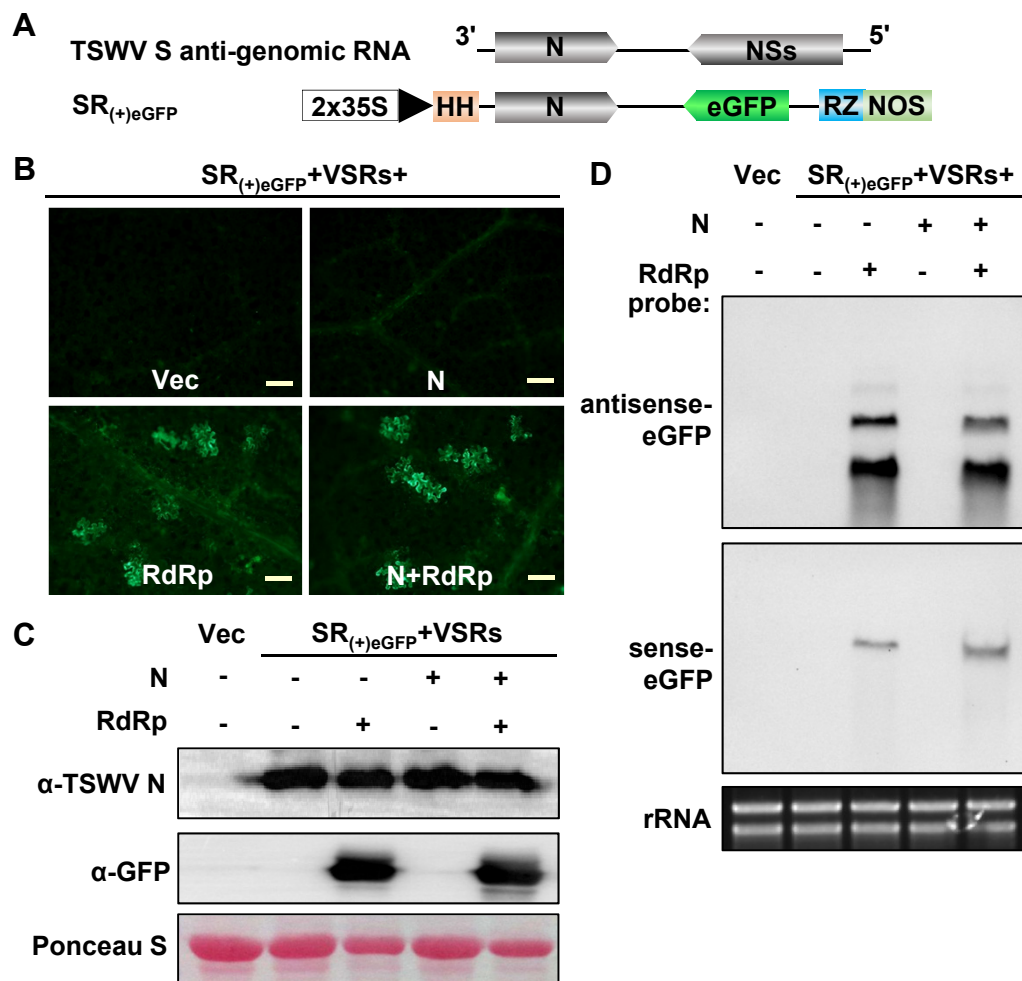


Fig. 3

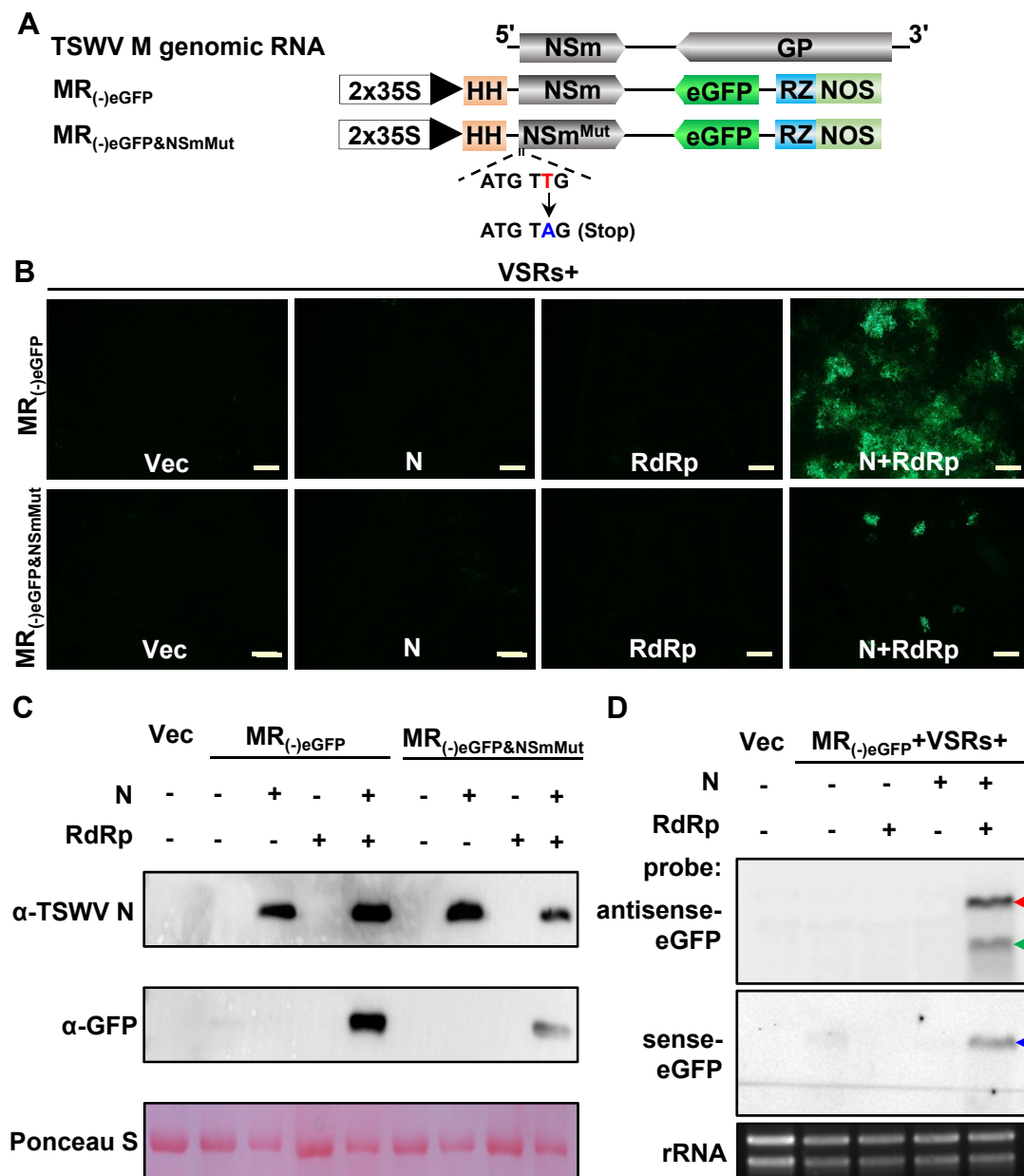
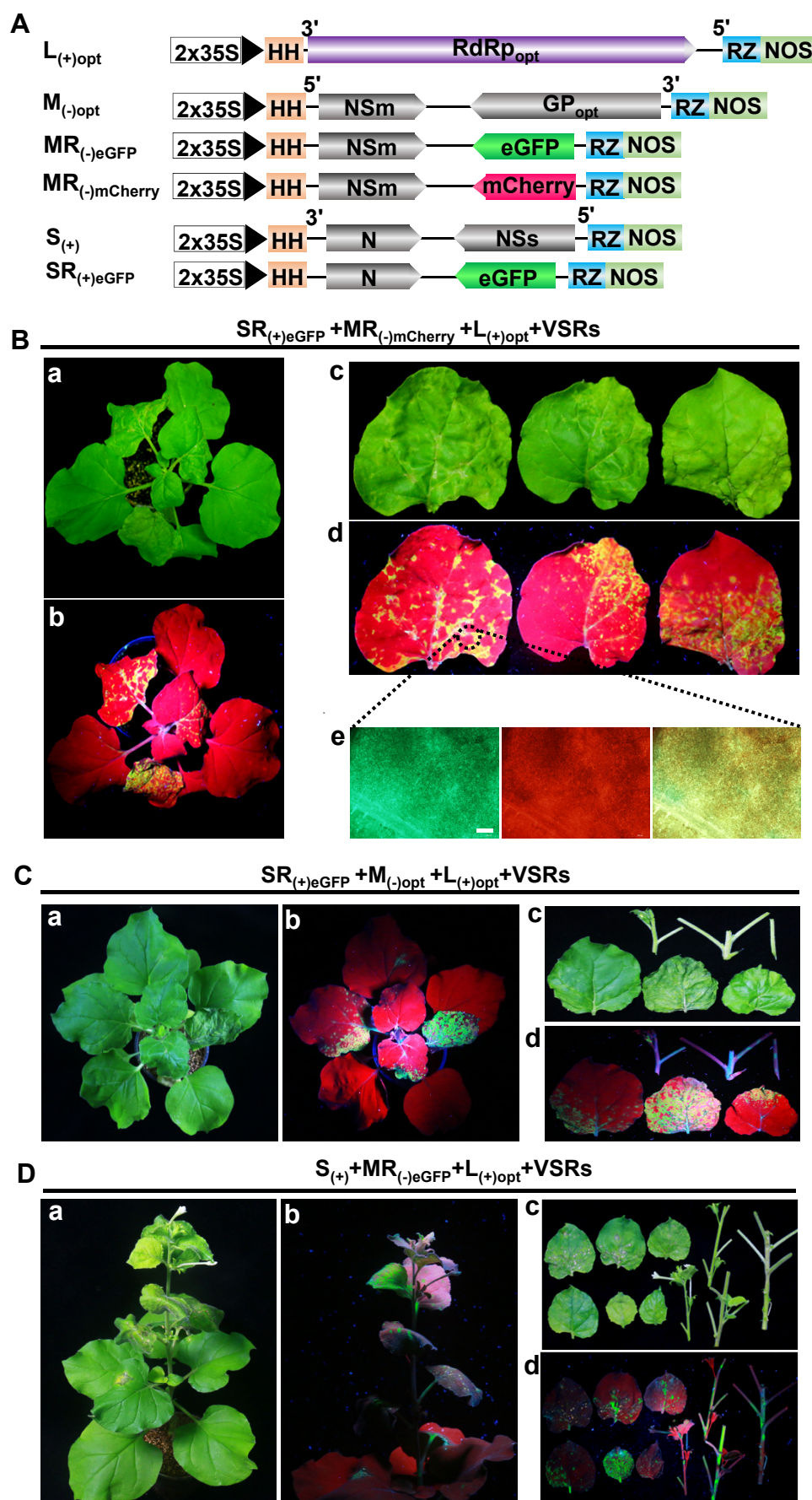
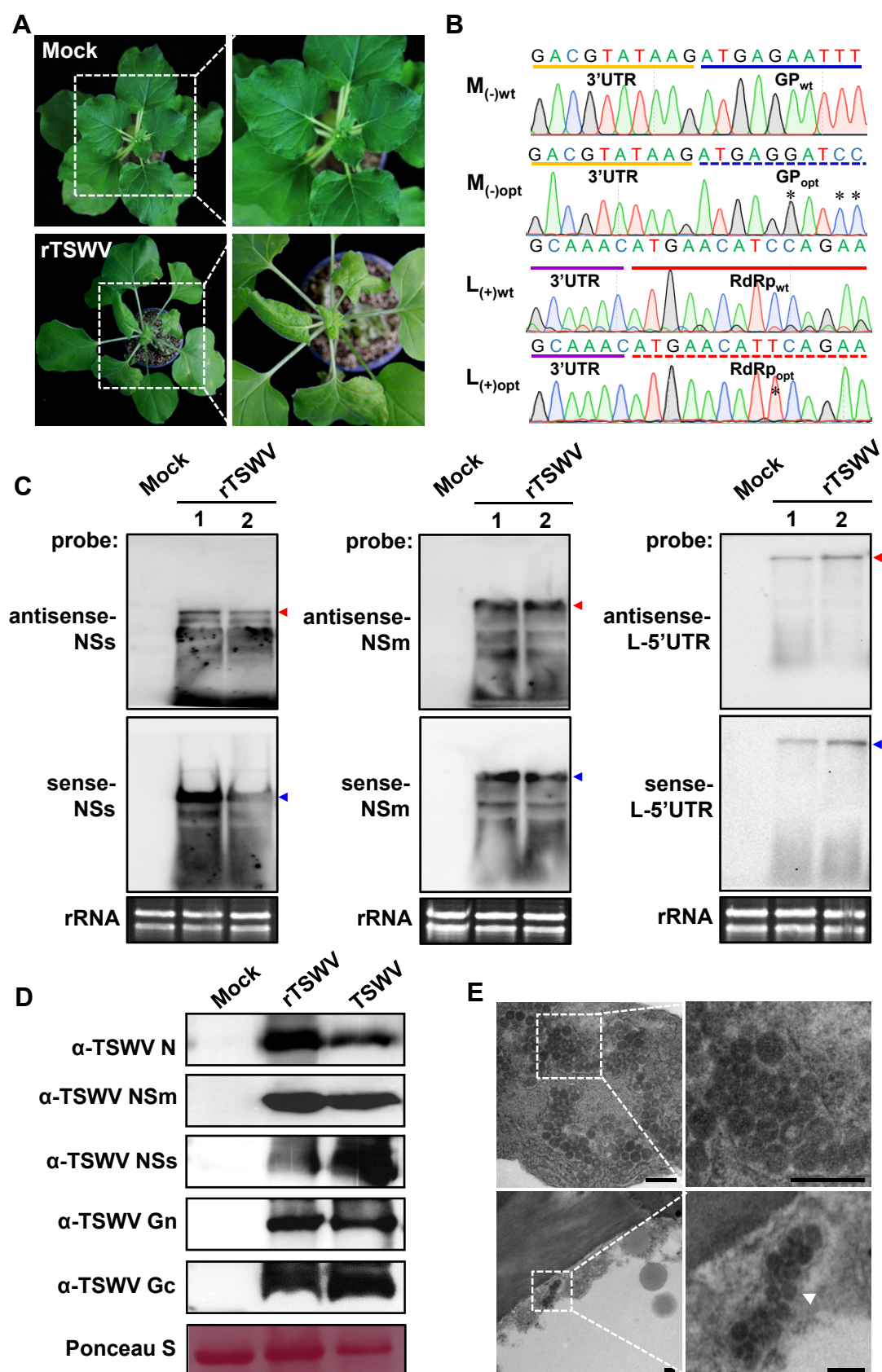


Fig. 4





**Fig. 5**



**Fig. 6**

# 1 **Supplementary Materials and Methods**

## 2 **Plasmid construction**

3 **Construction of RdRp, RdRp<sub>opt</sub>, N, NSs and VSRs.** The cDNA of the RdRp, N, and NSs  
4 genes was amplified from the total RNA of TSWV-lettuce isolate infected tissues and  
5 inserted into a binary vector pCambia2300 or pCXS<sub>N</sub> to generate p2300-RdRp<sub>wt</sub>, p2300-  
6 N, and pCXS<sub>N</sub>-NSs downstream a double 35S promoter (2×35S). The P19-HcPro-γb  
7 carrying three VSRs simultaneously in the pCB301 vector was kindly provided by Dr.  
8 Xianbing Wang in College of Biological Sciences of China Agricultural University. The  
9 codon usage and intron-splicing sites optimized sequence of RdRp ([SI Appendix, Fig. S9](#))  
10 was *de novo* synthesized by GenScript Biotech Corp (Nanjing, China) and was inserted  
11 into a binary vector pCambia2300 to generate p2300S-RdRp<sub>opt</sub> downstream a 2×35S  
12 promoter.

13 **Construction of full length TSWV genomic S<sub>(-)</sub>, M<sub>(-)</sub>, L<sub>(-)</sub>, and anti-genomic S<sub>(+)</sub>, M<sub>(+)</sub> and**  
14 **L<sub>(+)</sub> cDNA clones.** To generate constructs to express full length TSWV genomic RNA and  
15 antigenomic RNA S, M and L segments, total RNA extracted from TSWV-lettuce infected  
16 leaves of *N. benthamiana* plants was reverse transcribed into cDNA, followed by PCR  
17 amplification with specific primers ([SI Appendix, Table S3](#)) using Phanta Super-Fidelity  
18 DNA Polymerase (Vazyme Biotech, Nanjing, China). The PCR products were fused with  
19 a self-cleaving hammerhead (HH) ribozyme ([1](#)) and inserted into a binary expression  
20 vector pCB301-2×35S-RZ-NOS linearized by two restriction endonucleases *Stu* I and *Sma*  
21 I ([2](#)). The pCB301-2×35S-HH-S<sub>(-)</sub>-RZ-NOS [S<sub>(-)</sub>], pCB301-2×35S-HH-M<sub>(-)</sub>-RZ-NOS  
22 [M<sub>(-)</sub>], pCB301-2×35S-HH-L<sub>(-)</sub>-RZ-NOS [L<sub>(-)</sub>], pCB301- pCB301-2×35S-HH-S<sub>(+)</sub>-RZ-  
23 NOS [S<sub>(+)</sub>], 2×35S-HH-M<sub>(+)</sub>-RZ-NOS [M<sub>(+)</sub>] and pCB301-2×35S-HH-L<sub>(+)</sub>-RZ-NOS [L<sub>(+)</sub>]



cDNA clones were generated. The full length TSWV genomic S<sub>(-)</sub>, M<sub>(-)</sub> and L<sub>(-)</sub>, and anti-genomic S<sub>(+)</sub>, M<sub>(+)</sub> and L<sub>(+)</sub> were expressed downstream a double 35S promoter (2×35S) and flanked with a self-cleaving hammerhead (HH) ribozyme at 5'-terminus and a Hepatitis delta virus (HDV) ribozyme at 3'-terminus.

**Construction of TSWV SR<sub>(-)eGFP</sub>, SR<sub>(-)mCherry&eGFP</sub> and SR<sub>(+)eGFP</sub> minireplicons.** To generate SR<sub>(-)eGFP</sub>-genomic RNA minireplicon, the eGFP ORF was amplified and used to replace the N gene in the pCB301-2×35S-HH-S<sub>(-)</sub>-RZ-NOS by the *in vitro* recombination using the In-Fusion Cloning mixture (Clontech, Japan). The construct pCB301-2×35S-HH-SR<sub>(-)eGFP</sub>-RZ-NOS [SR<sub>(-)eGFP</sub>] was generated.

To generate SR<sub>(-)mCherry&eGFP</sub> in which the NSs and N genes in S gRNA were replaced with mCherry and eGFP, respectively, the mCherry ORF was amplified and used to exchange the NSs gene in the pCB301-2×35S-HH-SR<sub>(-)eGFP</sub>-RZ-NOS by recombination using In-Fusion Cloning mixture (Clontech). The construct pCB301-2×35S-HH-SR<sub>(-)mCherry&eGFP</sub>-RZ-NOS [35S:SR<sub>(-)mCherry&eGFP</sub>] was generated. The T7:SR<sub>(-)mCherry&eGFP</sub> minireplicon (pCB301-T7-HH-SR<sub>(-)mCherry&eGFP</sub>-RZ-NOS) controlled by T7 promoter was constructed by the same strategy as 35S:SR<sub>(-)mCherry&eGFP</sub>.

To generate antigenomic S<sub>(+)eGFP</sub>-minireplicon, the eGFP ORF was amplified and used to replace the NSs gene in the pCB301-2×35S-HH-S<sub>(-)</sub>-RZ-NOS by recombination using In-Fusion Cloning mixture (Clontech). The construct pCB301-2×35S-HH-SR<sub>(+)eGFP</sub>-RZ-NOS [SR<sub>(+)eGFP</sub>] was generated. The primers used above are listed in [SI Appendix, Table S3](#).

**Construction of SR<sub>(-)mCherry&eGFPΔ5'UTR</sub>, SR<sub>(-)mCherry&eGFPΔIGR</sub> and SR<sub>(-)mCherry&eGFPΔ3'UTR</sub> mutants.** To generate SR<sub>(-)mCherry&eGFPΔ5'UTR</sub>, the DNA copy of SR<sub>(-)mCherry&eGFP</sub> without 5'-

UTR (88 nt) was amplified from pCB301-2×35S-HH-SR<sub>(-)</sub>mCherry&eGFP-RZ-NOS and used to recombine with backbone vector of pCB301-2×35S-HH-SR<sub>(-)</sub>mCherry&eGFP-RZ-NOS by recombination using In-Fusion Cloning mixture (Clontech). The construct pCB301-2×35S-HH-SR<sub>(-)</sub>mCherry&eGFPΔ5'UTR-RZ-NOS [SR<sub>(-)</sub>mCherry&eGFPΔ5'UTR] was generated.

To generate SR<sub>(-)</sub>mCherry&eGFPΔIGR, the DNA copy of SR<sub>(-)</sub>mCherry&eGFP without IGR (550 nt) was amplified from pCB301-2×35S-HH-SR<sub>(-)</sub>mCherry&eGFP-RZ-NOS and used to recombine with the vector backbone from pCB301-2×35S-HH-SR<sub>(-)</sub>mCherry&eGFP-RZ-NOS by recombination using In-Fusion Cloning mixture (Clontech). The construct pCB301-2×35S-HH-SR<sub>(-)</sub>mCherry&eGFPΔIGR-RZ-NOS [SR<sub>(-)</sub>mCherry&eGFPΔIGR] was generated.

To generate SR<sub>(-)</sub>mCherry&eGFPΔ3'UTR, the DNA copy of SR<sub>(-)</sub>mCherry&eGFP without 3'-UTR (151 nt) was amplified from pCB301-2×35S-HH-SR<sub>(-)</sub>mCherry&eGFP-RZ-NOS and used to recombine with the vector backbone from pCB301-2×35S-HH-SR<sub>(-)</sub>mCherry&eGFP-RZ-NOS using the primer pair FMF48/P3382 by recombination using In-Fusion Cloning mixture (Clontech). The construct pCB301-2×35S-HH-SR<sub>(-)</sub>mCherry&eGFPΔ3'UTR-RZ-NOS [SR<sub>(-)</sub>mCherry&eGFPΔ3'UTR] was generated. The primers used above are listed in [SI Appendix, Table S3](#).

**Construction of TSWV MR<sub>(-)eGFP</sub>, MR<sub>(-)mCherry</sub> and MR<sub>(-)eGFPNSmMut</sub> minireplicons.** To generate MR<sub>(-)eGFP</sub> and MR<sub>(-)mCherry</sub> minireplicons, the eGFP and mCherry ORFs were amplified and used to replace the GP gene in pCB3012×35S-HH-M<sub>(-)</sub>-RZ-NOS, respectively, by recombination using In-Fusion Cloning mixture (Clontech). The constructs pCB301-2×35S-HH-MR<sub>(-)eGFP</sub>-RZ-NOS [MR<sub>(-)eGFP</sub>] and pCB301-2×35S-HH-MR<sub>(-)mCherry</sub>-RZ-NOS [MR<sub>(-)mCherry</sub>] was generated.

To generate MR<sub>(-)eGFPNSmMut</sub> in which a stop codon was introduced immediately after

the start codon of NSm, the NSm<sup>Mut</sup> was amplified and used to replace the wild type NSm sequence in pCB301-2×35S-HH-MR<sub>(-)</sub>eGFP-RZ-NOS by recombination using In-Fusion Cloning mixture (Clontech). The construct pCB301-2×35S-HH-MR<sub>(-)</sub>eGFPNSmMut-RZ-NOS [MR<sub>(-)</sub>eGFPNSmMut] was generated. All primers used above are listed in [SI Appendix, Table S3](#).

**Construction of full length *L*<sub>(+)opt</sub> and *M*<sub>(-)opt</sub> cDNA clones.** To generate full length *L*<sub>(+)opt</sub> cDNA clone, the sequence codon and intron-splicing sites optimized RdRp was amplified and used to replace the wild type RdRp sequence in pCB301-2×35S-HH-L<sub>(+)</sub>-RZ-NOS by recombination using the In-Fusion Cloning mixture (Clontech). The pCB301-2×35S-HH-L<sub>(-)opt</sub>-RZ-NOS [*L*<sub>(-)opt</sub>] was generated.

To generate full length *M*<sub>(-)opt</sub> cDNA clone, the codon and intron-splicing sites optimized GP gene was de novo synthesized by GenScript Biotech Corp (Nanjing, China) ([SI Appendix, Fig. S10](#)) and used to replace the wild type GP sequence in pCB301-2×35S-HH-M<sub>(-)</sub>-RZ-NOS by the *in vitro* recombination using In-Fusion Cloning mixture (Clontech). The pCB301-2×35S-HH-M<sub>(-)opt</sub>-RZ-NOS [*M*<sub>(-)opt</sub>] was generated. The primers used above are listed in [SI Appendix, Table S3](#).

## Plant material and virus source

Six to eight weeks of *Nicotiana benthamiana* was used in all agroinfiltration assay. *N. benthamiana* plants were grown in a growth chamber setting at 25 °C, a 16 h light and 8 h dark photoperiod (3). The TSWV isolate from asparagus lettuce (TSWV-LE) was used in this study (GenBank accession number: KU976396 for S, JN664253 for M and KU976394 for L) (4). The TSWV-LE isolate was maintained on *N. benthamiana*. For long-term storage, the infected new leaves of *N. benthamiana* were kept in an 80 °C refrigerator.

## *Agrobacterium* infiltration

Recombinant plasmids were electroporated into *Agrobacterium tumefaciens* strain GV3101 and agroinfiltrations were performed essentially as described (5, 6). *A. tumefaciens* cells were resuspended by agroinfiltration buffer [10 mM MgCl<sub>2</sub>, 10 mM MES (pH 5.6) and 100 μM acetosyringone] adjusted to an optical density OD<sub>600</sub> of 1.0 and incubated for 2 to 3 h in dark at room temperature. Equal volumes of *Agrobacterium* cultures (final concentration OD<sub>600</sub>=0.2) harboring the p2300-N, p2300-RdRp, pCB301-derived reporter or full-length infectious clone vector(s), were mixed with one volume of bacterial mixture (final concentration OD<sub>600</sub>=0.05) containing the NSs and P19-HcPro-γb. The *Agrobacterium* cultures were infiltrated into fully expanded leaves of 6-7 leaf stage *N. benthamiana* plants using 1 mL needleless syringes.

#### **Immunoblot analysis**

Total protein was extracted from 1 g *Agrobacterium*-infiltrated leaf patches, healthy or TSWV-infected *N. benthamiana* systemic leaves in a 1 mL extraction buffer [10 % (v/v) glycerol, 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 10 mM dithiothreitol, 2 % (w/v) polyvinylpyrrolidone, 0.5 % (v/v) Triton X-100 and 1× protease inhibitors cocktail] (7). Protein samples were separated by SDS-PAGE gels, transferred to PVDF membranes (GE Healthcare, UK), blocked with 5 % skim milk solution and incubated with a polyclonal antiserum specific to the TSWV N, NSm, NSs, Gn, Gc, GFP, mCherry or T7 RNA pol at room temperature for 1 h or overnight at 4 °C and washed three times. After incubation in a secondary antibody containing HRP-conjugated goat anti-rabbit (1:10000) for 1 h, the blots were detected using the ECL Substrate Kit (Thermo Scientific, Hudson, NH, USA). To evaluate protein loading, the blots were stained with Ponceau S.

#### **Northern blot analysis**

For Northern blot analysis of TSWV gRNAs, agRNAs or viral mRNA transcripts, total

118 RNAs were extracted from *Agrobacterium*-infiltrated leaf patches, healthy or TSWV-  
119 infected systemic leaves using an RNAprep Pure Plant Kit (Tiangen Biotech, Beijing,  
120 China), respectively. DIG-labeled specific probes for sense or antisense GFP, NSs, NSm,  
121 L-5'UTR was synthesized by DIG High Prime RNA labeling kit (Roche, Basel,  
122 Switzerland). The total RNAs were separated on 1 % formaldehyde agarose gels and  
123 transferred to Hybond-N+ membranes (GE Healthcare, UK) (8). The membrane blots were  
124 hybridized with a DIG-labeled specific probe and detected using a DIG-High Prime  
125 Detection Starter Kit II (Roche), following the manufacturer's protocol.

#### 126 **RT-PCR and sequencing analysis**

127 To detect the virus in systemic leaves of *N. benthamiana* infected with  
128 SR<sub>(+)</sub>eGFP+MR<sub>(-)</sub>mCherry+L<sub>(+)</sub>opt, S<sub>(+)</sub>+MR<sub>(-)</sub>eGFP+L<sub>(+)</sub>opt, SR<sub>(+)</sub>eGFP+M<sub>(-)</sub>opt+L<sub>(+)</sub>opt or rTSWV  
129 recovered from the full-length cDNA clones, total RNAs were extracted from systemic  
130 symptoms plant leaves. First-strand cDNAs were synthesized using M-MLV Reverse  
131 Transcriptase (Promega, USA). RT-PCRs were performed to detect the SR<sub>(+)</sub>eGFP,  
132 MR<sub>(-)</sub>mCherry, MR<sub>(-)</sub>eGFP, S<sub>(+)</sub>, M<sub>(-)</sub>opt and L<sub>(+)</sub>opt minigenome and genomic RNA using their  
133 specific-primers. The PCR products were inserted into a pMD19-T vector (Takara, Dalian,  
134 China) and sequenced by Sanger dideoxy-mediated chain-termination DNA sequencing  
135 method at Sangon Biotech (Shanghai, China). The primers used in this study are listed in  
136 [SI Appendix, Table S3](#).

#### 137 **Fluorescence microscopy**

138 The agro-infiltrated *N. benthamiana* leaves were examined for fluorescence expression  
139 using an OLYMPUS IX71-F22FL/DIC Inverted Fluorescence Microscope (OLYMPUS,  
140 Tokyo, Japan) with a green or red barrier filter. The leaf sample was fixed in water on a

microslider under a coverslip to detect the eGFP and mCherry fluorescence, respectively. Fluorescence images were processed using ImagePro (OLYMPUS, Tokyo, Japan) and Adobe (San Jose, CA, USA) Photoshop programs.

# **Electron microscopy**

Small tissues (1 mm × 4 mm) were excised from leaves of the *N. benthamiana* with infected rTSWV rescued from the full-length infectious clones. The sample tissues were fixed in 2.5 % glutaraldehyde and 1 % osmium tetroxide dissolving into 100 mM phosphate buffer (pH 7.0) as described by Li *et al* (5, 9) and then embedded in Epon 812 resin as instructed by the manufacture (SPI-EM, Division of Structure Probe, Inc., West Chester, USA). Ultrathin sections (70 nm) were mounted on formvar-coate grids and then stained with uranyl acetate for 10 min followed by lead citrate for 10 min. The stained sections were examined under a transmission electron microscope (TEM; H-7650, Hitachi, Japan).

# **Imaging GFP in infected plant by hand-held UV lamp**

GFP fluorescence in leaves was monitored with a hand-held 100 W, long-wave UV lamp (UV Products, Upland, CA, USA) and the leaves were photographed using a Canon EOS 70D digital camera (Canon, Japan) with a 58 mm UV filter.

# **References**

1. Herold J & Andino R (2000) Poliovirus requires a precise 5' end for efficient positive-strand RNA synthesis. *J Virol* 74(14):6394-6400.
2. Shen Y, *et al.* (2014) A versatile complementation assay for cell-to-cell and long distance movements by cucumber mosaic virus based agro-infiltration. *Virus Res* 190:25-33.
3. Voinnet O & Baulcombe DC (1997) Systemic signalling in gene silencing. *Nature* 389(6651):553.
4. Jiang L, *et al.* (2017) Occurrence and diversity of Tomato spotted wilt virus isolates breaking the Tsw resistance gene of Capsicum chinense in Yunnan, southwest China. *Plant Pathol* 66(6):980-989.
5. Wang Q, *et al.* (2015) Rescue of a plant negative-strand RNA virus from cloned cDNA: insights into enveloped plant virus movement and morphogenesis. *PLoS Pathog* 11(10):e1005223.
6. Ganesan U, *et al.* (2013) Construction of a Sonchus yellow net virus minireplicon: a step toward

reverse genetic analysis of plant negative-strand RNA viruses. *J Virol* 87(19):10598-10611.

7. Li J, *et al.* (2019) A plant immune receptor adopts a two-step recognition mechanism to enhance viral effector perception. *Mol Plant* 12(2):248-262.

8. Feng MF, *et al.* (2018) Identification of Strawberry vein banding virus encoded P6 as an RNA silencing suppressor. *Virology* 520:103-110.

9. Kong L, Wu J, Lu L, Xu Y, & Zhou X (2014) Interaction between Rice stripe virus disease-specific protein and host PsbP enhances virus symptoms. *Mol Plant* 7(4):691-708.

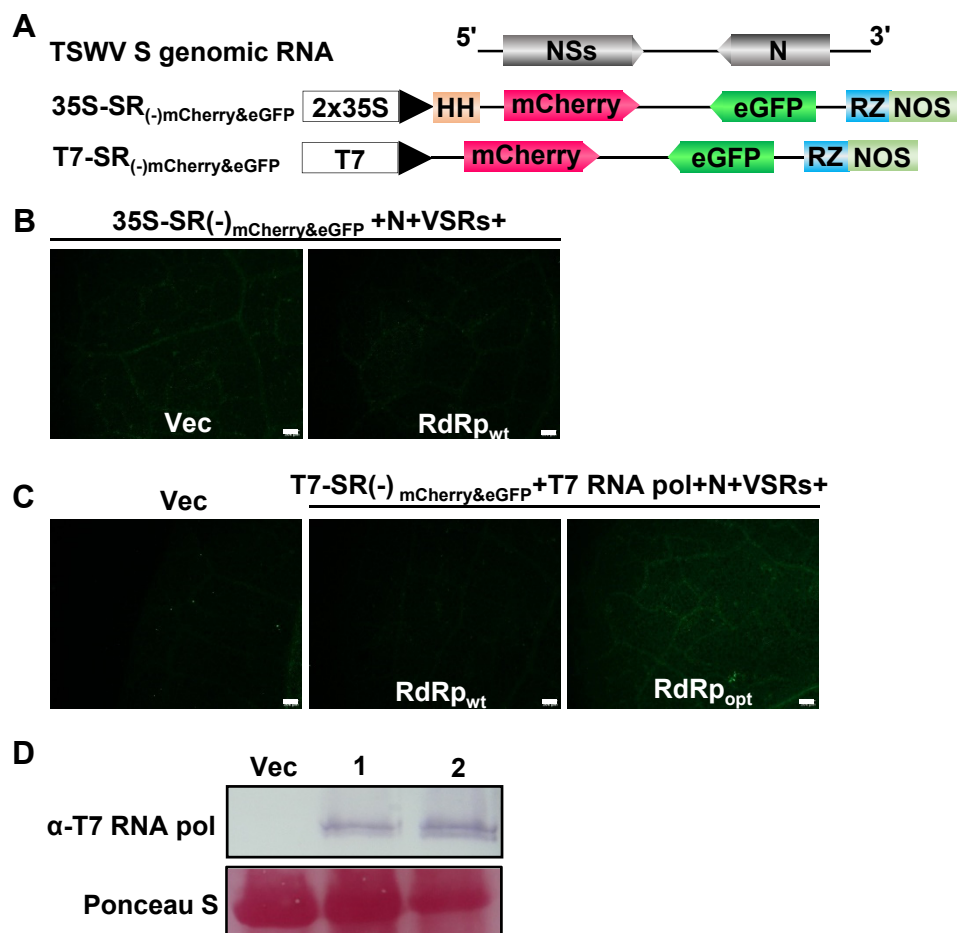
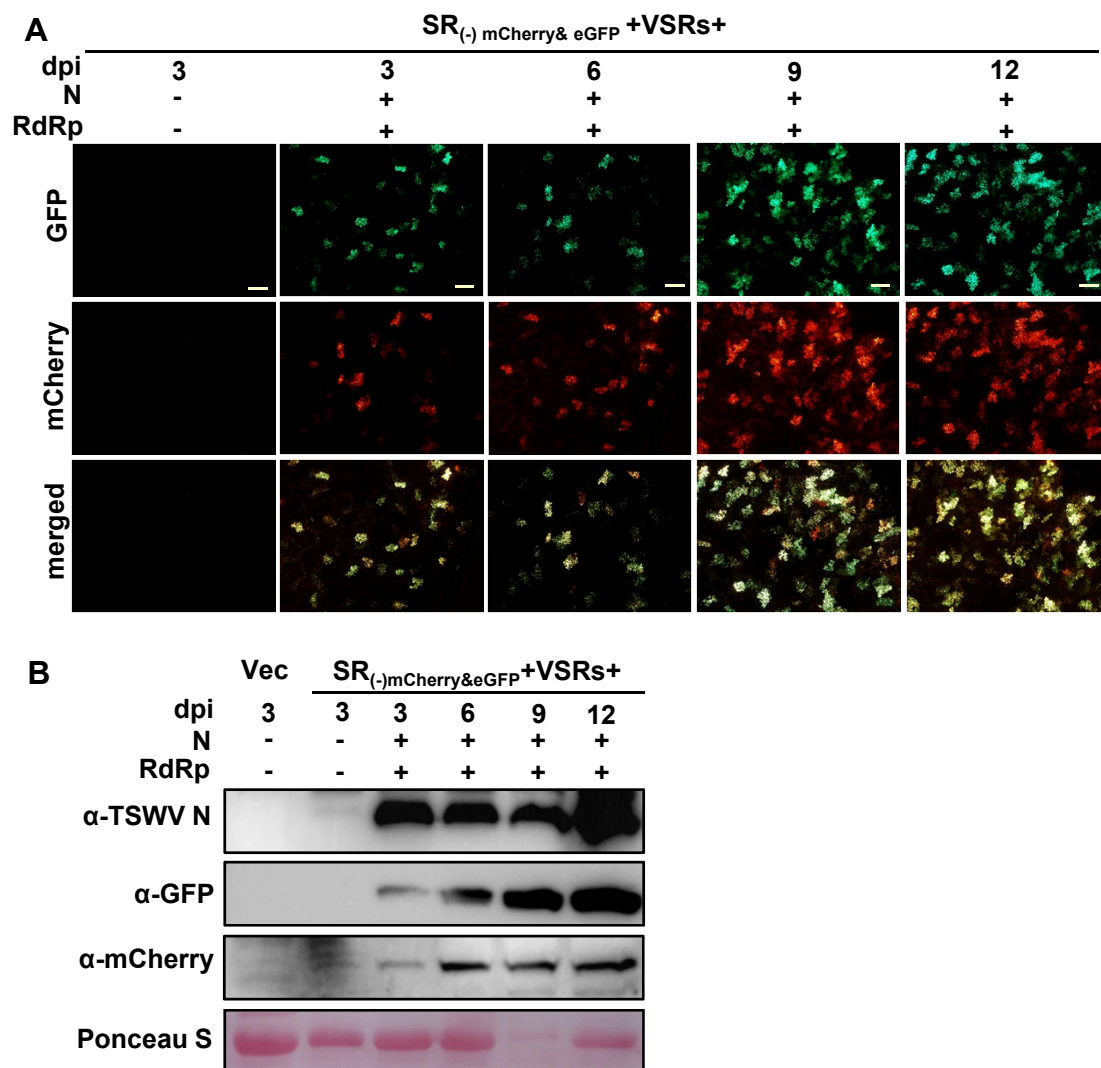


Fig. S1





**Fig. S2**

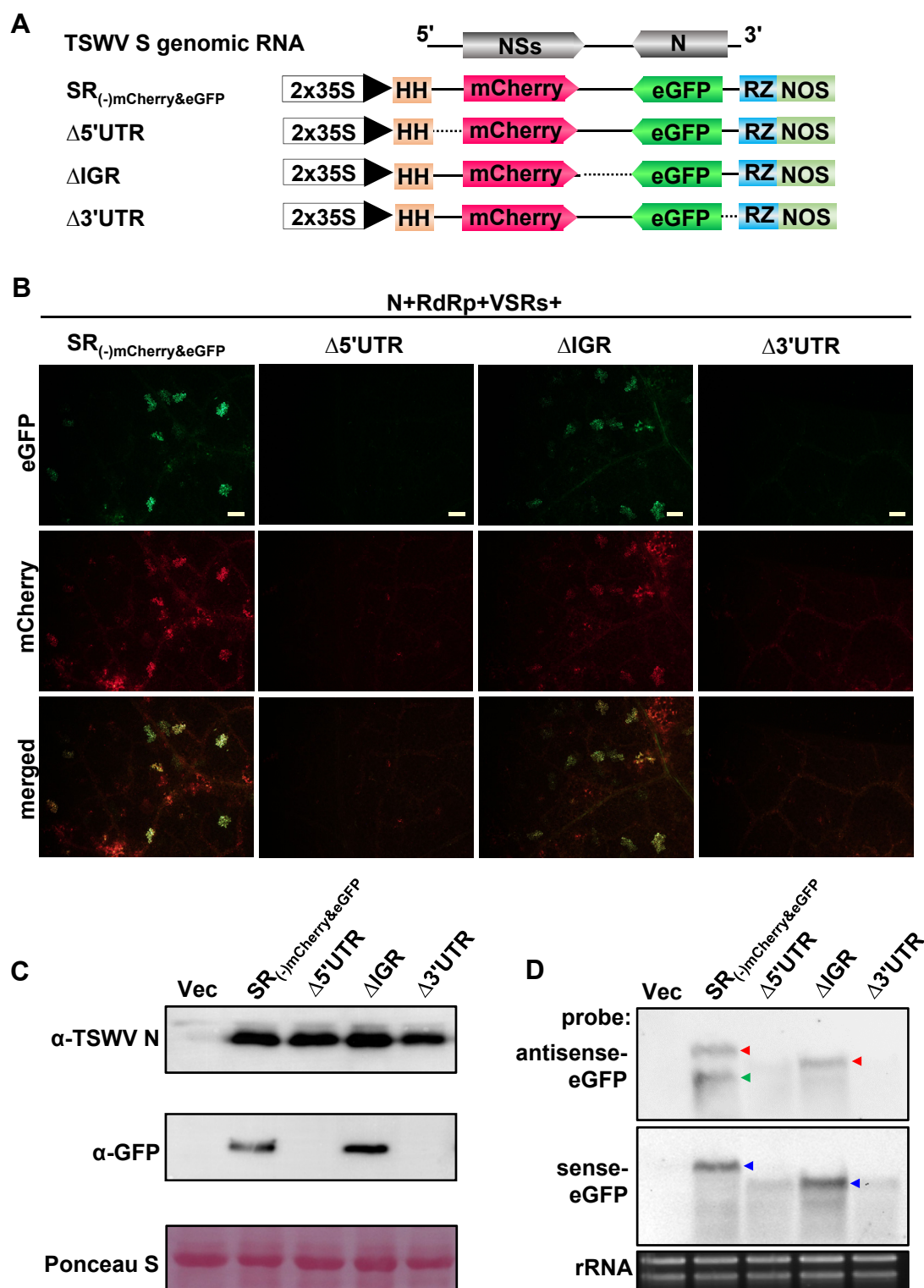
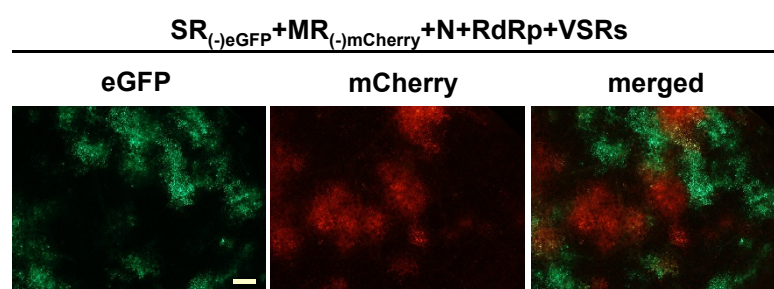
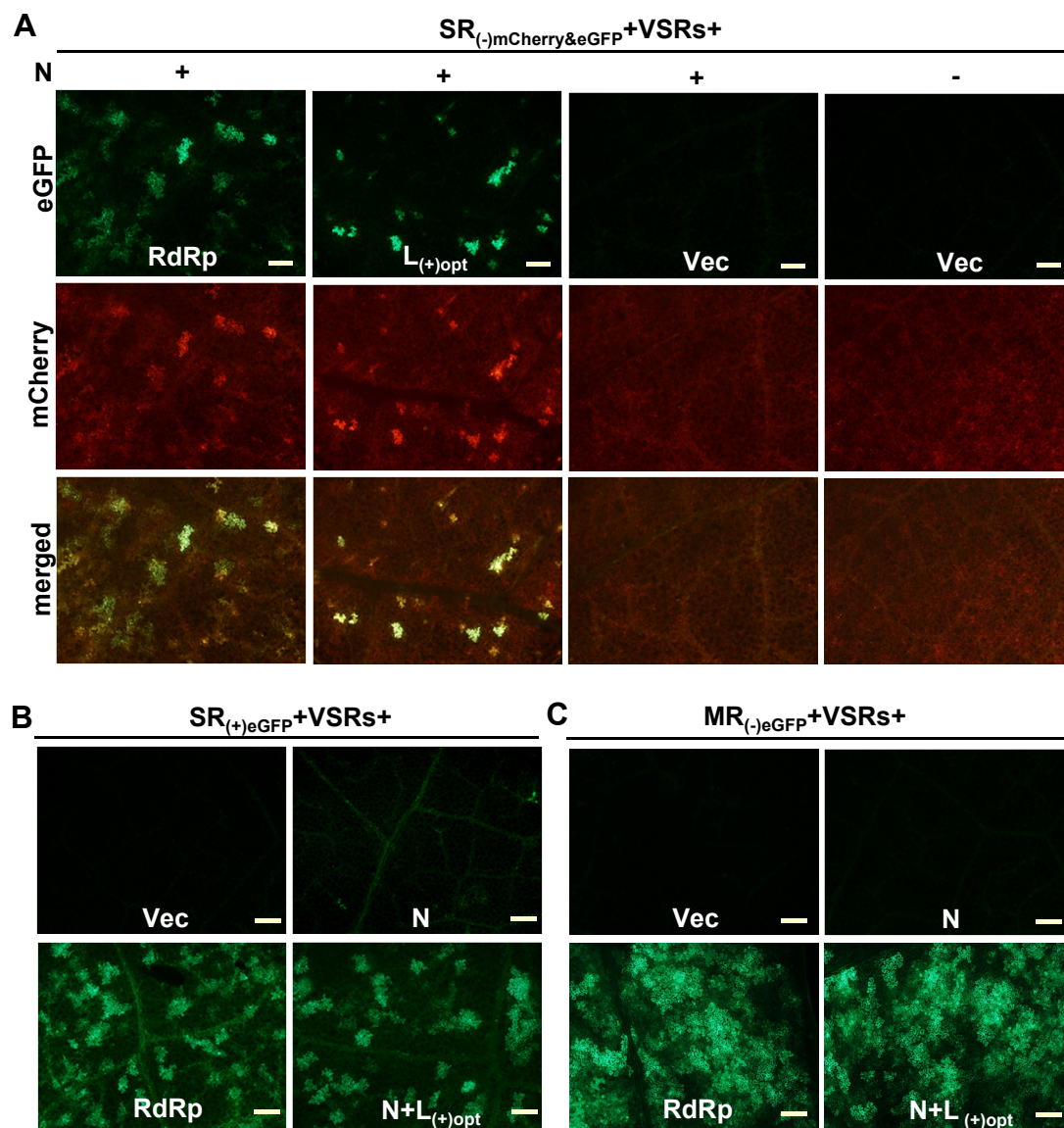


Fig. S3



**Fig. S4**



**Fig. S5**

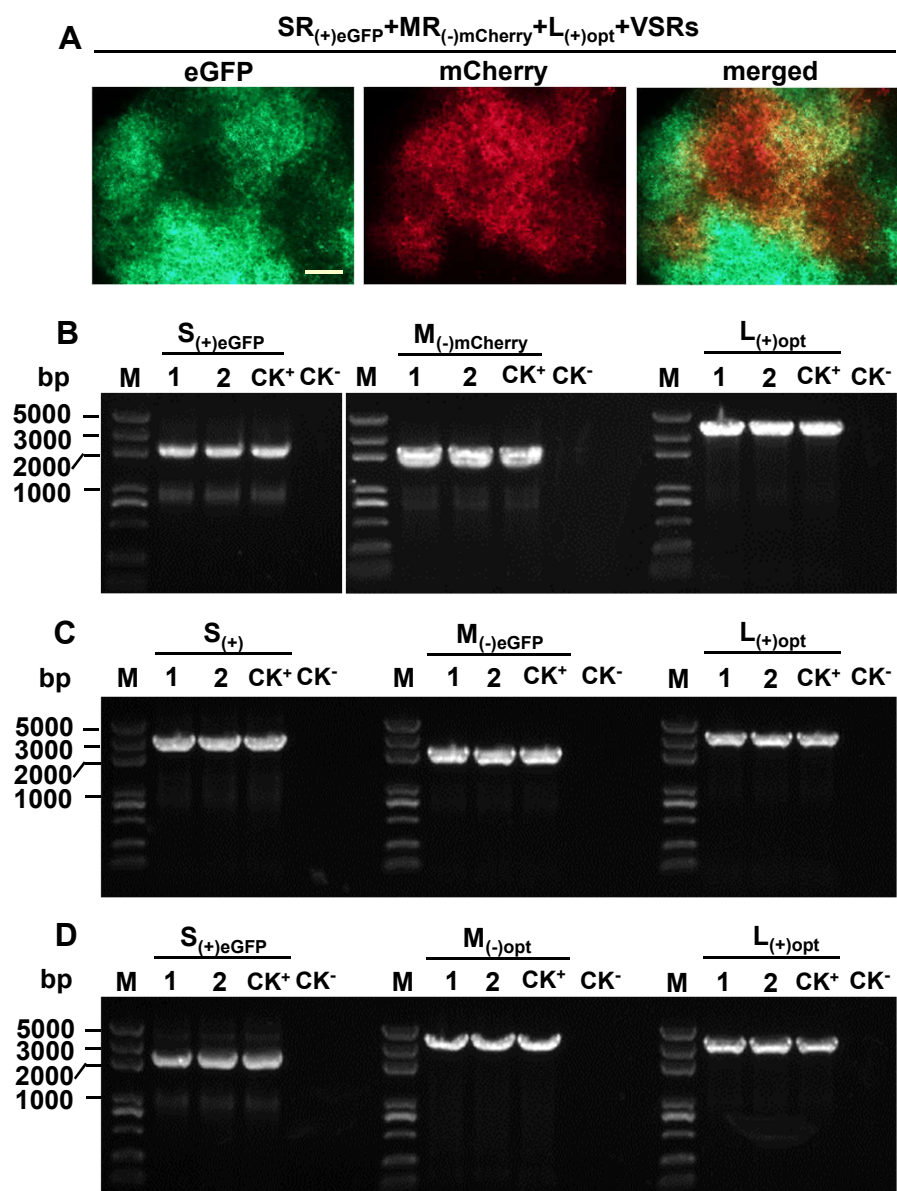


Fig. S6



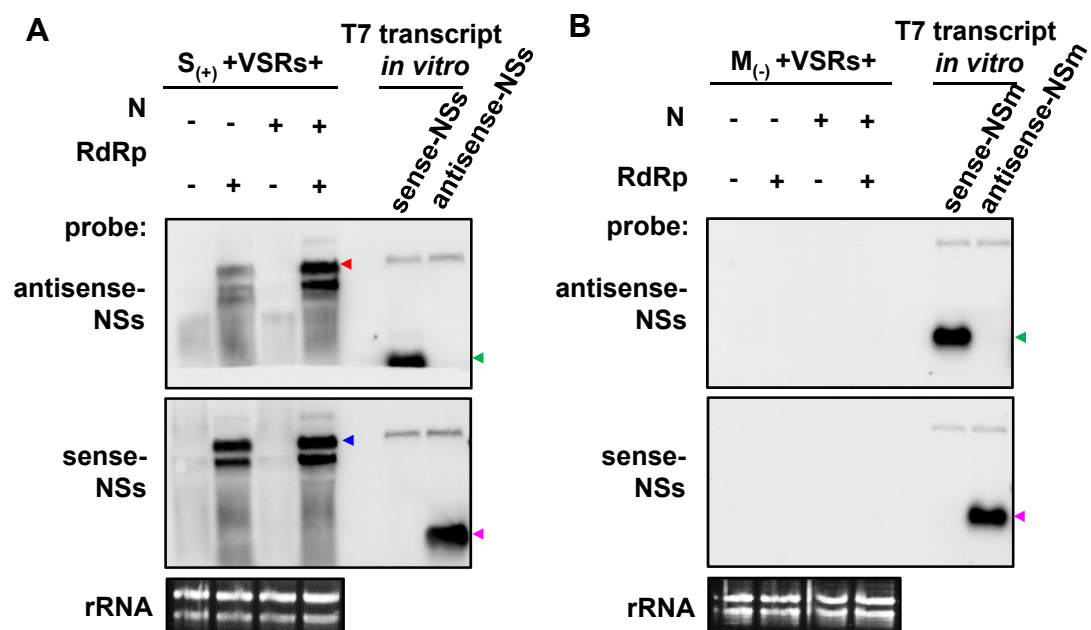
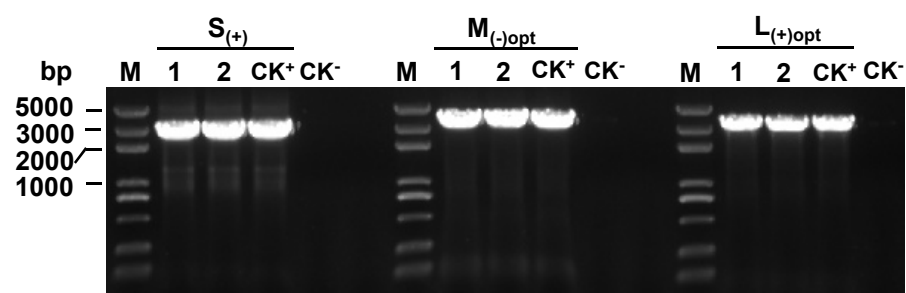


Fig. S7



**Fig. S8**

**Fig. S9. Optimized RdRp gene sequence used in the study.**

ATGAACATTCAGAAGATCCAAAAGCTGATCGAGAACGGCACCACCTTGCTGCTTTCTATTGAGGATTGCGTGGGCAGCAA  
CTACGATCTTGCTCTTGATCTGCACAAGCGGAACTCCGATGAGATTCTTGAGGACGTGATCATCAACAACAACGCCAAGA  
ACTACGAGACTATGCGTGAGCTGATCGTGAAGATCACTGCTGATGGTGAGGGTCTGAACAAAGGTATGGCTACCGTGGAC  
GTGAAGAACTGTCTGAGATGGTGTCTCTGTTTCGAGCAGAAGTACCTTGAGACTGAGCTTGCTAGGCACGATATCTTCGG  
CGAGCTTATTTCTAGGCACCTGAGGATCAAGCCGAAGCAGAGATCTGAGGTTGAGATTGAGCACGCTCTGAGAGAGTAC  
CTGGACGAGCTTAACAAGAAGTCTGTCATCAACAAGCTGAGCGACGATGAGTTCGAGCGGATCAACAAAGAATATGTGG  
CCACCAACGCTACCCCGGACAATTACGTTATCTACAAAGAGAGCAAGAACAGCGAGCTGTGCCTGATCATCTACGACTGG  
AAGATCTCTGTGGATGCCAGGACTGAGACTAAGACCATGGAAGTACTACAAGAACATCTGGAAGTCCTTCAAGGACAT  
CAAGGTGAACGGCAAGCCTTTCTTGAGGATCATCTGTGTTCTGTGAGCATCGTGATCCTGAAGCCTATTGCTGGCATGC  
CTATTACCGTGACCTCTAGCAGAGTGCTCGAGAAGTTTGAGGACTCTCCTTCTGCACTTCACGGCGAGAGGATTAAGCAC  
GCTAGGAACGCTAAGCTGCTGAACATTTCTCACGTGGGTGAGATCGTGGGTACTACTCCTACTGTGGTGAGGAATACTA  
CGCTAACACCCAGAAGATCAAGTCCGAGGTTAGGGGTATCCTGGGTGATGATTTCCGGCAGCAAGGACGTGTTCTTCTCTC  
ACTGGACCTCCAAGTACAAAGAGCGGAACCTACCGAGATCGCTTACTCTGAGGATATCGAGCGTATCATCGACAGCCTT  
GTGACCGACGAGATCACCAAGAAGAGATTATCCACTTCCTGTTCGGCAACTTCTGCTTCCACATCGAGACAATGAACGA  
CCAGCACATTGCCGACAAGTTCAAGGGCTATCAGTCTCTTGTCATCAACCTGAAGATCGAGCCTAAGGTGGACCTGGCTG  
ATCTTAAGGATCACCTGATTGAGAAGCAGCAGATTTGGGAGAGCCTGTACGGTAAGCACCTCGAGAAGATTATGCTGCGG  
ATCCGTGAAAAAGAAGAAGAAAGAGATCCCGGACATCACCACCGCCTTCAATCAAAATGCTGCCGAGTACGAAG  
AGAAGTACCCGAAGTCTTACCAACGACCTGTGAGAGACAAAGACCAACTTCTCTATGACCTGGTCACCGAGCTTCGA  
GAAAATCGAGCTGTCTAGCGAGGTGGACTACAACAATGCCATTATCAACAAGTTCCGTGAGAGCTTCAAGTCCAGCAGCA  
GGGTGATCTACAACAGCCCTTACAGCTGCATTAACAACAGACCAACAAGGCCCGGGATATCACTAACCTTGTGAGGCTT  
TGCCTGACCGAGCTTTCTTGCGATACCACCAAGATGGAAAAGCAAGAGCTTGAGGACGAGATCGACATCAACACCGGTA  
GCATTAAGGTGCGAGCGGACCAAGAAAAGCAAAGAGTGGAAACAAGCAGGGCTCCTGCCTTACTAGGAACAAGAACGAGTT  
CTGCATGAAGGAAACCGGCCGAGAGAACAAGACCATCTACTTCAAGGGTCTTGCCGTGATGAACATCGGCATGTCTCTA  
AGAAGCGGATTCTGAAAAAGAGGAAATCAAAGAGAGGATCAGCAAGGGCCTCGAGTACGATACTTCTGAGAGACAGGC  
TGATCCGAACGACGACTACAGCAGCATCGATATGTCATCACTGACCCATATGAAGAAGCTCATCCGGCAGGATAACGAGG  
ACTCACTTTCTTGGTGGAGCGAATCAAGGACAGCTTGTTCTGTGCTTCAACCGCGATATCAGAGAAGAGGGCAAGAT  
CACCAGCGTGTAACAATTACGCTAAGAACCCGAGTGCCTGTACATCCAGGATTCTGTGCTTAAGACCGAGCTGGAAA  
CCTGCAAGAAAATCAACAACTGTGCAACGATCTGGCCATCTACCACTACAGCGAGGACATGATGCAGTTCTCCAAGGGT  
TTGATGGTGGCCGATCGGTACATGACCAAAAGAGTCTTCAAGATCCTGACCACCGCCAATACCAGCATGATGTGCTTGC  
TTTCAAAGGCGACGGTATGAACACTGGTGGTTCTGGTGTCTCTTACATTGCCCTGCACATCGTGGATGAGGATATGTCCG  
ATCAGTTCAACATCTGCTACACCAAGAGATCTACAGCTACTTCCGGAACGGCTCCAACCTACATCTACATCATGAGGCCGC  
AGAGGCTTAACAGGTGAGGCTTCTTTCACTGTTCAAGACCCCTTCTAAGGTGCCAGTTTGCTTCGCCAGTTTACAGCAA  
AAGGCCAACGAAATGGAAAAATGGCTGAAGAAACAAGGACATTGAGAAGGTCAACGTGTTACAGCATGACCATGACCGTGA  
AGCAGATCCTGATCAACATCGTGTCTCCAGCGTGATGATTGGCACCGTGACTAAGCTTTCTCGGATGGGCATCTTCGAC  
TTCATGAGGTACGCTGGTTTCTTGCCGCTGTCCGACTACTCCAACATCAAAGAGTACATCCGGGACAAGTTCCGACCCGA  
TATTACCAATGTGGCCGATATCTACTTCGTGAACGGGATCAAGAACTGCTGTTCCGGATGGAAGATTTGAACCTGAGCA



CCAACGCAAAGCCTGTTGTGGTGGATCACGAGAACGATATCATCGGTGGCATCACCGACCTGAACATCAAGTGCCCTATT  
ACTGGTTCTACCTGTGACCTTGAGGACCTGTATAACAAATGTGTACCTCGCCATCTACATGATGCCGAAGTCTCTGCAT  
AACCACGTGCACAACCTTACCAGCCTGCTTAATGTTCTGTGAGTGGGAGCTGAAGTTCCGGAAAGAGCTTGGCTTCA  
ACATTTTCGAGGACATCTACCCGAAGAAAGCCATGTTTCGATGACAAGGACCTCTTCAGCATTAAACGGCGCTCTTAACGTG  
AAGGCCCTGAGCGATTACTACCTGGGTAACATCGAGAATGTGGGCCTGATGAGGTCCGAGATTGAGAACAAAGAGGACT  
TCCTGTCTCCGTGCTACAAGATCTCTACCCTGAAGTCCAGTAAGAAGTGCAGCCAGAGCAACATCATCAGCACTGATGAG  
ATCATTGAGTGCCTGCAGAACGCAAAGATTACAGGACATCGAAAACCTGGAAGGGCAACAACCTGGCTATTATCAAGGGCCT  
GATCCGGACCTACAACGAGGAAAAAGATCGGCTGGTTGAGTTCTTCGAGGATAACTGCGTGAACAGCCTGTACCTGGTC  
GAGAAGCTTAAAGAGATCATTAACAGCGGCAGCATCACCGTGGGAAAGTCTGTGACTAGCAAGTTCATCCGTAACAATCA  
CCCCTGACCGTGGAAACCTACCTCAAGACTAAGCTGTACTATCGGAACAACGTGACCGTGTCTGAAGTCTAAGAAGGTG  
AGCGAGGAAGTGTACGACCTCGTTAAGCAGTTCACAAACATGATGGAAATCGACCTGGACTCTGTGATGAACCTTGTAA  
GGGTACTGAGGGGAAGAAGCACACCTTCTTGCAAGTGTGAGTTCGTGATGAGCAAGGCCAAGAATGTGACCGGTTCT  
GTGGATTTCCTCGTGAGCGTGTTCGAAAAAGATGCAGAGGACCAAGACCGACCGTGAGATCTACCTGATGAGCATGAAGG  
TGAAGATGATGCTGTACTTCATCGAGCATACCTTCAAGCACGTGGCCAGTCTGATCCTTCTGAGGCTATTAGCATCAGCG  
GCGACAACAAGATTAGGGCTCTGTCTACCTTAGCCTGGACACCATTACCAGCTACAACGACATCTCAACAAGAATAGC  
AAGAAGTCTCGGCTGGCTTTCCTGAGCGTGATCAATCTAAGTGGTCCGCTTCTGACCTGACCTACAAGTACGTGCTGGC  
CATCATTTCTGAACCCGATTCTTACTACTGGCGAGGCCTCTCTTATGATCGAGTGCATTCTGATGTACGTGAAGCTGAAGAA  
AGTGTGCATCCCGACCGACATTTTCTTGAACCTTAGGAAGGCTCAGGGCACCTTCGGTCAAAACGAAACCGCTATTGGTC  
TGCTGACCAAGGGACTTACCACCAACACTTACCCGGTGTCTATGAACTGGCTGCAGGGTAACCTGAACTACCTCTCTTCT  
GTGTACCACTCCTGCGCTATGAAGGCTTACCACAAGACTCTCGAGTGTCTATAAGGACTGCGACTTTCAGACCCGGTGGAT  
CGTTCACCTCTGATGATAACGCAACCAGCCTGATCGCTTCAGGTGAGGTTGACAAGATGCTGACCGACTTCTCCTCTTCAT  
CTCTGCCTGAGATGCTGTTTCAGATCCATCGAGGCTCACTTCAAGTCTTCTGATCACAACCTCAACCCCAAGAAGTCATAC  
GCCAGCTCAAGCGAGGTGAGTTCATTCTGAGAGGATTGTGAACGGCGCTATCATCCACTTTACTGCAGGCATCTTGC  
TAACTGCTGCACCGAGTCTCTCACATCTCCTACTTCGATGACCTGATGTCCCTGTCTATCCACGTGACCATGTTGCTGAG  
AAAGGGCTGCCCTAATGAGGTGATCCCTTTTGCTTACGGTGTGTGCAAGTGCAGGCTCTCTCTATCTACTCTATGCTGC  
CTGGTGAGGTGAACGACAGCATCCGGATCTTAAAGAAGCTGGGCGTCAGCCTCAAGTCCAACGAGATTCTACTAATCATG  
GGCGGTTGGCTGACCTCTCCTATTGAGCCTTTGTCTATTCTGGGCCCCAGCAGCAACGACCAGATTATCTACTACAACGT  
GATCCGGGATTTCCTGAACAAGAAATCCCTGGAAGAGGTGAAGGACTCCGTGTCCTCATCTTCTTACCTGCAGATGAGGT  
TCAGGGAACTCAAGGGCAAGTACGAGAAGGGTACTCTGGAAGAGAAGGACAAAAAGATGATCTTCCTTATCAACCTCTTC  
GAGAAGGCCAGCGTGTCCGAGGATAGTGATGTGCTTACCATCGGGATGAAGTTCAGACCATGCTGACCCAGATCATCAA  
GCTGCCCAACTTCATCAACGAGAACGCCTTGAACAAGATGAGCAGCTACAAGGACTTCTCCAAGCTGTACCCCAACCTCA  
AGAAGAACGAGGATCTGTACAAGTCCACCAAGAACCTCAAAATCGACGAGGACGCTATCCTCGAGGGTGTGAGCTTTAT  
GAGAAGATCGCCAGCAGCCTCGAGATGGAATCTGTGCACGACATCATGATCAAGAACCCGGAAACCATTTCTGATCGTCC  
GCTGAACGATAGGGACTTTCTGCTGTCTCAGCTGTTTCATGTACACCTCTCCGTCTAAGAGGAACCAAGCTGTCTAATCAGT  
CTACCGAGAAGCTGGCTCTTGATAGGGTGTGAGATCTAAGGCTAGGACCTTCGTGGACATCTCCTCTACCGTTAAGATG  
ACCTATGAAGAGAACATGGAAAAAAGATCTGGAGATGCTCAAGTTCGATCTCGACAGCTACTGCAGCTTCAAGACTTG  
CGTGAACCTGGTGTGATCAAGGATGTGAACTTCTCCATGCTTATCCGATCCTCGACTCTGCTTATCCTTGCGAGTCTAGGAA  
GCGGGACAACCTACAACCTCCGTTGGTTCCAGACTGAGAAGTGGATCCCTGTTGTTGAGGGTTCTCCTGGACTTGTGGTG

ATGCATGCTGTGTACGGGTCTAACTATATCGAGAACCTCGGCCTTAAGAACATCCCGCTTACCGACGACTCTATCAACGTG  
CTGACTTCTACCTTCGGCACCAGGTCTGATTATGGAAGATGTCAAGAGCCTGGTGAAGGGCAAAGACTCATTTGAGACAGA  
GGCCTTCAGCAACTCTAACGAGTGCCAGAGACTTGTGAAGGCCTGCAACTACATGATCGCTGCTCAGAACAGGCTCCTG  
GCTATCAATACCTGCTTTACCCGGAAGTCTTTCCCGTTCTACTCCAAGTTCAATCTTGGCAGGGGCTTCATCAGCAACACC  
CTTGCTTTGCTGTCCACCATCTACAGCAAAGAGGAATCCTACCACTTCGTGTCCACCGCCTCTTACAAGCTGGATAAGAC  
TATCCGGACCGTGATCTCTGCACAGCAGGATATGAACCTGGAAAAGATCCTGGATACCGCCGTGTACATCAGCGACAAGC  
TTCAGTCTTTGTTCCCGACCATCACCAGAGAGGACATCGTTCTCATTCTCCAGAACGTGTGCCTGGACTCCAAGCCTATT  
TGGCAGTCTTTGGAGGACAAGATGAAGAAGATTAAACAACAGCACCGCCAGCGGCTTACCCTGTCTAATGTGATTCTGAG  
CCACAACCTCCGAGCTGAACACCATCCAGAAAACAGATCGTGTGGATGTGGAACATGGGCCTTTGCTCTCATAGGACCCTGG  
ATTTCTGTATCCGTTACATCAGGCGGTCCGATGTGAGATACGTCAAGACTGAGGAACAGGACGAGAGCGGGAACCTACATT  
AGCGGAACCATGTACAAGATTGGCATCATGACCCGGTCTTGCTACGTGCAGTTGATCGCATCAGATCAGGATGTGGCTGT  
GTCTCTTAGGACCCCTTTCGAGATTCTCAACGAGAGGGATTACCTGTTTCGACACCTACCGTGAGTCCATCGAAAAGCTGC  
TGCAAAAGTTTATGTTTCGACAAGGTCAACATTATCAAGTCTAAGCAGCCGAGATTGTGTTCTCGAGCCAGGTGATGCT  
TGCATTAGGATGACCACCGACAACAAAATGATCGTCAAGGTAAACGCCACCAGCAGGCAGATTAGGCTTGAGAACGTTAA  
GCTGGTGGTGAAGATTAAAGTATGAGAACGTGAATCCGACGTGTGGGACATCATTGAGAGCCAGAAGTCTCTCGTTCTC  
CGGCTTCTGAAGTTGGTGAGTGCTTCAGCGACATGTATAAGACCGCTGACAGCGAGACTGAGACAATCAAGACTGTGA  
AGAACCGGCTGATGACCTCTCTGACCTTCATTGAGGCTTTCGGCAACCTCAGCCAGCAGATCAAAGAAATCGTGGACGAT  
GACATCAGGGAAACCATGGACGAGTTCCTCATGAACATCCGTGATACCTGCCCTTGAGGGTCTTGAGAATTGCAAGAGCGT  
GGAAGAGTACGACTCCTACCTTGATGAGAACGGGTTCACGATACCGTCGAGCTGTTTCGAGAATCTTCTGAGGACCCAC  
GACAACCTTCGAGAACGAGTACTCTCCACTGTTTCAGCGAGATCGTGGATAAGGCTAAGCAGTACACCAGGGATCTCGAGG  
GCTTTAAAGAAATCCTGCTGATGCTGAAGTACTCTCTGATCAACGACGCTCCGGGTTCAGTCAATAGAGCTACTGGTA  
TGCACGCCGTTGAGCTGATGGCTAAGAAACACATTGAGATCGGCGAGTTCAACCTGCTGGGTATGATTACGCTTATCAAG  
GCTTTCGAGACTTGCCACAACAACGACTCCATTCTCAACCTGGCCAGCCTTAGGAATGTGCTGTCTAGGACTTACGCTAC  
TTTCGGCAGGCGGATCAGGTTGAATCACGATCTGGATCTGCAGAAACACCTTATGGAAAAGAGCTACGACTTCAAGACCT  
TGGTGCTCCCCGAGATTAAGCTCTCTGAGCTGAGCAGGGAAATCCTGAAAGAGAACGGCTTCGTTATCTCCGGCGAGAA  
CCTTAAGATGGACAGGTCCGATGAAGAGTTCGAGGGGCTCGCTTCATTCAATGTGCTTAGGTTGGACGAGGAAGAGATG  
TACGAGGGGCTGATCAAAGAAATGAAGATCAAGCGGAAGAAGAAGGGCTCTCTGTTCCCTGCTAATACCTGCTTCTGTG  
CGAGTTGATCAAGTTCCTGATCGGCGGTATTAAGGGCACCAGCTTCGATATTGAGACTCTGCTCCGGAACCTCATTAGGC  
CGGATATCTTCTTACCGACAGGCTTGGTAGGCTCTCTTCTTCTGTGCCTGCTCTTAAGGTGTACGCCACTGTGTACATGG  
AGTACAAGAACGTCAACTGCCCTCTGAACGAGATCGCCGATTCTCTTGGGGTTACCTGAAGCTCACCAAGTCCAAGAG  
CAAAGAACACTTCTTGAGCGGCAGGGTGAAGAAGGCTCTTATTAGTTGAGGGACGAGCAGTCCAGGACCAAAAAGCTC  
GAGGTCTACAAGGATATCGCCAACCTTCCTTAGCAGGCACCTCTTTGCTGTCTGAAAAGACACTGTACGGCCGGTACAC  
CTACAGCGATATCAACGATTACATCATGCAGACCCGGGAAATCATCCTCTCTAAGATCTCCGAGTTGGATGAGGTGGTGG  
AAACTGACGAGGACAACCTCCTGCTCTCTTACCTTAGGGGCGAAGAGGATGCTTTCGATGAGGACGATTCTGATGAGGA  
AGAGGACACCGATTAG

**Fig. S10. Optimized GP gene sequence used in the study.**

ATGAGGATCCTGAAGCTTCTTGAGCTGGTGGTGAAGGTGAGCCTGTTTACCATTGCTCTGTCTCTGTGCTTCTGGCCTT  
CCTTATTTTCAGGGCTACCGACGCTAAGGTCGAGATCATTAGAGGCGATCATCCTGAGGTGTACGACGACTCTGCTGAGA  
ATGAAGTTCCTACCGCTGCTAGCATTACAGCGGAAGGCTATTCTTGAGACTCTGACCTCTCTGATGCTCGAGTCTCAAACCT  
CCTGGAACCAGGCAGATTCTGTGAGGAAGAGTCTACCATTCTTATCTTCGCTGGCAGCACTACCCAGAAGATTATCTCCGT  
TAGCGACCTGCCTAACAACCTGCCTGAACGCTTCTCTGAGTGCAGATCAAGGGCATCAGCACCTACAACGTTTACT  
ACCAGGTGAGAACAAACGGCGTGATCTACTCTTGCGTGTGAGATTCTGCTGAGGGCCTTGAGAAGTGCAGAACCTCTCT  
TAACCTGCCGAAGCGGTTCTCTAAGGTGCCAGTGATTCTTATCACCAGCTGGACAACAAGCGGCACTTCTCTGTGGGCA  
CCAAGTTCTTCATTAGCGAGTCTCTGACCCAGGACAACCTACCCGATTACCTACAACAGCTACCCCTACCAACGGAACCGTG  
TCTCTTCAGACCGTTAAGCTGTCTGGCGATTGCAAGATCACCAAGAGCAACTTCGCTAACCCGTATACCGTGAGCATTAC  
CTCTCCAGAGAAGATCATGGGCTACCTGATCAAGAAGCCTGGCGAAAACGCTGAGCACAAGGTGATCTCATTCTCCGGCT  
CTGCTTCCATTACCTTACCGAAGAGATGCTGGATGGTGAGCACAACTTCTCTGCGGTGATAAGTCTGCTAAGATCCCT  
AAGACCAACAAGCGTGTGAGGGACTGCATCATCAAGTACAGCAAGAGCATCTACAAGCAGACCGCCTGCATCAACTTCTC  
TTGGATTAGGCTGATCCTGATCGCCCTGCTGATCTACTTCCCTATTAGATGGCTGGTGAACAAGACCACCAAGCCGCTTTT  
CTGTGGTACGATCTGATCGGCCTGATTACTTACCCAATCCTGCTGCTGATTAAGTGCCTGTGGAAGTACTTTCCGTTCAA  
GTGCAGCAACTGCGGCAACCTGTGCATTATTACTCACGAGTGACCAAGATCTGCATCTGCAACAAGAGCAAGGCCAGC  
AAAGAACACTCTAGCGAGTGCCGATCCTGAGCAAAGAAACCGATCACGACTACAACAAGCACAAGTGGACCAGCATGG  
AATGGTTCCACCTTATCGTGAACACCAAGCTCAGCTTCAGCCTGCTTAAGTTCGTGACCGAGATTCTGATCGGTCTGGTG  
ATCCTTAGCCAGATGCCTATGTCTATGGCTCAGACTACCCAGTGCCTTAGCGGTTGCTTTTATGTTCTGGTTGCCCTGTG  
CTGGTGACCTCTAAGTTTGAAAAGTGCCCTGAGAAGGACCAAGTGCTACTGCAACGTGAAAGAGGACAAGATCATCGAGT  
CCATCTTCGGCACCAACATCGTGATTGAGGGTCTAACGACTGCATCGAGAACCAGAATTGTGTGGCTCACCCGAGCATC  
GACAACCTGATTAAGTGTAGACTGGGCTGCGAGTACCTGGACCTGTTTAGAAAACAAGCCTCTGTACAACGGCTTCAGCGA  
CTACACCGGTTCTTCACTTGGTCTTACCTCTGTGGGACTGTACGAGGCTAAGAGGCTTAGGAACGGCATCATCGACTCTT  
ACAACCGGACCGATAAGATCAGCGGTATGATCGCTGGTGACAGCCTGGATAAGAACGAGACTTCTATCCCCGAGAACATC  
CTGCCAAGGCAGTCTCTGATTTTCGACTCTGTGGTGGATGGCAAGTACCGGTATATGGTTGAGCAGAGCCTTCTTGGAGG  
TGGTGGAACTGTGTTTCATGCTGAACGATAAGACCTCTGAGAAGGCCAAGAAGTTCGTCTATCATCAAGAGCGTGGGC  
ATCCACTACGAGGTGTCAGAGAAGTATACCACCGCTCCTATTAGTCTACCCACACCGATTCTACTCTACCTGCACCGGT  
AATTGCGATACCTGCAGAAAGAACCAGGCTCTGACAGGCTTTTACGACTTCTGCATTACCCCTACCTCTTACTGGGGTTG  
TGAAGAGGCTTGGTGCTTCGCTATTAAACGAGGGTGCTACTTGGCGCTTCTGCCGAATATCTACGACATGGACAAGAGCT  
ACCGGATCTACAGCGTGCTGAAGTCTACTATCGTTGCCGACGTGTGCATCTCCGGTATTCTTGGTGGACAGTGCTCTAGG  
ATTACCGAAGAGGTTCCATACGAGAACGCTCTGTTCAGGCTGATATTCAGGCTGATCTGCACAACGATGGCATCACCAT  
TGGTGAGCTTATTGCTCACGGACCGGACAGCCATATCTACTCCGGTAACATTGCTAACCTGAACGACCCGGTGAAGATGT  
TCGGTCATCCTCAGCTTACTCATGACGGCGTGCCAATCTTACCAAGAAAACCTTGAGGGCGACGACATGTCTTGGGAT  
TGTGCTGCTATCGGCAAGAAGTCCATCACCATCAAGACCTGCGGTTACGACACTTACAGGTTACGGTCTGGTCTTGAGCA  
GATCTCTGACATCCCCATCAGCTTCAAGGACTTCAGCTCATTCTTCTGGAAGAGCTTACGCTTGGGGAAGCTGAAGA  
TCGTGGTGGATCTGCCTAGCGATCTTTTCAAGGTTGCACCTAAGCGGCCGTCTATCACTTCTACTAGGCTTAACTGCAAC  
GGCTGCCTTCTTTGTGGTCAGGGCCTTTCTTGCATCCTCGAGTTCTTCTCTGATCTGACCTTCAGCACCGCCATCTCTATC

GATGCTTGCTCTTTGTCTACCTACCAGCTGGCTGTGAAGAAGGGCAGCAACAAGTACAACATCACCATGTTCTGCAGCGC  
AAACCCGGACAAGAAAAAGATGACTCTGTACCCTGAGGGCAACCCCGATATTCTGTTGAGGTGCTCGTGAACAACGTG  
ATCGTTGAGGAACCTGAGAACATCATCGATCAGAACGACGAGTACGCTCACGAGGAACAGCAGTACAACAGCGATTTCATC  
TGCTTGGGGATTCTGGGATTACATCAAGTCCCCGTTCAACTTCATTGCCAGCTACTTCGGCAGCTTCTTCGATACAATCAG  
GGTGATCCTGCTTATCGCCTTCATCTTCCTGGTTATCTACTTCTGCAGCATCCTGACCACCATCTGCAAGGGTTACGTGAA  
GAACAAGTCCTACAAGAGCCGGTCCAAGATCGAGGATGATGACGACTCAGAGATTAAGGCCCCGATGCTGATGAAGGAC  
ACTATGACTAGAAAGGCGGCCTCCGATGGATTCTCTCATCTTGTTAG

**Table S1. The predicted intron splicing sites of wild-type RdRp gene.**

Position (bp)	Putative splice site	Sequence	Score*	Intron GC*	Activations**		
					Alt./Cryptic	Constitutive	Confidence**
69	Alt. isoform/cryptic donor	TGAGGATTGCgtgggcagca	4.835	0.529	0.954	0.035	0.963
175	Alt. isoform/cryptic donor	GAGACTATGCgtgagctgat	4.841	0.514	0.933	0.051	0.946
205	Constitutive donor	ACTGCTGATGgtgaggtct	12.212	0.500	0.370	0.546	0.322
223	Alt. isoform/cryptic donor	CTGAACAAAGgtatggctac	10.886	0.500	0.802	0.142	0.823
261	Alt. isoform/cryptic donor	GTCTGAGATGgtgtctctgt	5.341	0.486	0.882	0.086	0.903
280	Alt. isoform/cryptic acceptor	gttcgagcagAAGTACCTTG	5.730	0.514	0.943	0.055	0.942
281	Alt. isoform/cryptic donor	TCGAGCAGAAgtaccttgag	5.847	0.500	0.816	0.135	0.834
336	Constitutive acceptor	ttattctagGCACCTGAGG	4.932	0.486	0.212	0.770	0.725
579	Alt. isoform/cryptic acceptor	tggatgccagGACTGAGACT	4.087	0.500	0.696	0.295	0.576
602	Alt. isoform/cryptic donor	CCATGGAAAgtactacaag	6.059	0.457	0.939	0.044	0.953
642	Alt. isoform/cryptic donor	GGACATCAAGgtgaacggca	7.873	0.514	0.821	0.135	0.835
681	Alt. isoform/cryptic donor	TCCTGTGTTcgtgagcatcg	6.292	0.529	0.839	0.123	0.853
738	Alt. isoform/cryptic acceptor	tgacctctagCAGAGTGCTC	3.060	0.500	0.836	0.157	0.812
741	Alt. isoform/cryptic acceptor	cctctagcagAGTGCTCGAG	3.393	0.514	0.762	0.227	0.703
831	Alt. isoform/cryptic donor	CATTTCACgtgggtcaga	5.665	0.500	0.943	0.042	0.955
835	Alt. isoform/cryptic donor	TCTCACGTGGgtcagatcgt	6.026	0.500	0.936	0.048	0.949
843	Alt. isoform/cryptic donor	GGGTCAGATCgtgggtacta	5.056	0.514	0.903	0.074	0.918
864	Alt. isoform/cryptic donor	TCCTACTGTGgtgaggaact	11.320	0.500	0.901	0.072	0.920
906	Alt. isoform/cryptic donor	CAAGTCCGAGgttaggggta	10.127	0.514	0.709	0.226	0.682
913	Alt. isoform/cryptic donor	GAGGTTAGGGgtatcctggg	4.945	0.514	0.953	0.032	0.967
971	Alt. isoform/cryptic donor	GGACCTCCAgtacaagag	5.665	0.514	0.934	0.049	0.948
1185	Alt. isoform/cryptic donor	CGAGCCTAAGgtggacctgg	4.643	0.514	0.716	0.215	0.700
1207	Alt. isoform/cryptic acceptor	tgatcttaagGATCACCTGA	2.743	0.500	0.596	0.388	0.349
1249	Alt. isoform/cryptic donor	AGCCTGTACGtaagcacct	11.990	0.457	0.579	0.329	0.431
1361	Alt. isoform/cryptic donor	ACGAAGAGAAgtaccgcaac	5.393	0.529	0.904	0.069	0.923
1388	Alt. isoform/cryptic donor	CCAACGACCTgtcagagaca	5.572	0.500	0.910	0.068	0.925
1421	Alt. isoform/cryptic donor	CTATGACCTGgtcaccgagc	5.097	0.471	0.952	0.036	0.962
1492	Alt. isoform/cryptic donor	AACAAGTTCCgtgagagctt	6.085	0.500	0.565	0.376	0.334
1515	Alt. isoform/cryptic acceptor	agtccagcagCAGGGTGATC	2.527	0.486	0.940	0.057	0.939
1542	Alt. isoform/cryptic acceptor	gcccttacagCTGCATTAAC	3.273	0.486	0.526	0.454	0.136

1587	Alt. isoform/cryptic donor	CACTAACCTTgtgagcttt	7.336	0.514	0.944	0.040	0.958
1672	Constitutive donor	ATCAACACCGgtagcattaa	5.999	0.500	0.395	0.516	0.233
1740	Constitutive acceptor	gcettactagGAACAAGAAC	7.612	0.529	0.146	0.841	0.827
1801	Alt. isoform/cryptic acceptor	ctactcaagGGTCTTGCCG	3.996	0.471	0.717	0.270	0.624
1889	Alt. isoform/cryptic donor	AGGGCCTCGAgtacgatact	5.278	0.500	0.956	0.032	0.966
2006	Alt. isoform/cryptic donor	CACTTTCTTGgtgcgagcga	6.544	0.529	0.958	0.031	0.968
2125	Alt. isoform/cryptic acceptor	gtacatccagGATTCTGTGC	6.304	0.514	0.555	0.432	0.222
2205	Alt. isoform/cryptic acceptor	accactacagCGAGGACATG	4.878	0.457	0.847	0.147	0.827
2230	Alt. isoform/cryptic acceptor	gttctccaagGGTTTGATGG	2.346	0.471	0.692	0.290	0.580
2321	Constitutive acceptor	gctttcaagGCGACGGTAT	6.287	0.457	0.385	0.587	0.344
2326	Alt. isoform/cryptic donor	AAAGGCGACGgtatgaacac	7.959	0.486	0.760	0.181	0.761
2487	Constitutive donor	GCTTAACCAggtgagcttc	14.109	0.500	0.246	0.676	0.637
2512	Alt. isoform/cryptic acceptor	actgttcaagACCCCTTCTA	4.626	0.514	0.933	0.062	0.933
2523	Alt. isoform/cryptic donor	CCCTTCTAAGgtgccagttt	10.939	0.471	0.614	0.306	0.501
2545	Alt. isoform/cryptic acceptor	cttgcgccagTTCAGCAAAA	6.490	0.529	0.692	0.300	0.566
2550	Alt. isoform/cryptic acceptor	cccagttcagCAAAAAGGCC	4.578	0.500	0.800	0.194	0.758
2601	Alt. isoform/cryptic donor	CATTGAGAAggtcaacgtgt	6.294	0.500	0.944	0.041	0.956
2664	Constitutive acceptor	tgttctccagCGTGATGATT	6.507	0.486	0.462	0.524	0.117
2720	Alt. isoform/cryptic donor	ACTTCATGAGgtacgtggt	9.451	0.543	0.706	0.226	0.680
2765	Alt. isoform/cryptic donor	ACATCAAAGgtacatccgg	4.583	0.500	0.944	0.041	0.956
2942	Constitutive donor	TGAACATCAAggtccctatt	6.540	0.500	0.357	0.573	0.378
3217	Alt. isoform/cryptic donor	TACTACCTGGgtaacatcga	6.561	0.500	0.885	0.083	0.906
3292	Alt. isoform/cryptic acceptor	gtgtacaagATCTCTACCC	3.251	0.514	0.960	0.038	0.961
3307	Alt. isoform/cryptic acceptor	taccctgaagTCCAGTAAGA	3.209	0.500	0.930	0.067	0.928
3310	Alt. isoform/cryptic donor	CTGAAGTCCAgtaagaagtgt	9.173	0.471	0.712	0.218	0.694
3370	Constitutive acceptor	gtgcctgcagAACGCAAAGA	3.539	0.500	0.477	0.500	0.047
3557	Alt. isoform/cryptic donor	CCGTGGGAAAggtctgtgact	6.027	0.500	0.919	0.059	0.935
3642	Alt. isoform/cryptic donor	TCGGAACAACgtgaccgtgc	5.533	0.500	0.919	0.061	0.934
3666	unclassified donor	GTCTAAGAAggtgagcgagg	13.918	0.500	0.418	0.490	0.000
3745	Alt. isoform/cryptic donor	ATGAACCTTGgtaagggtac	11.753	0.500	0.609	0.303	0.502
3751	Alt. isoform/cryptic donor	CTTGTAAGGgtactgaggg	6.065	0.500	0.952	0.033	0.966
3784	Constitutive acceptor	cttctgcagATGCTTGAGT	7.888	0.514	0.201	0.782	0.742
3816	Alt. isoform/cryptic donor	GGCCAAGAATgtgaccggtt	6.207	0.543	0.936	0.047	0.950

3840	Alt. isoform/cryptic donor	GGATTTCCTCgtgagcgtgt	5.915	0.514	0.899	0.079	0.912
3880	Alt. isoform/cryptic donor	AAGACCGACCgtgagatcta	5.588	0.457	0.542	0.380	0.299
3906	Alt. isoform/cryptic donor	GAGCATGAAGgtgaagatga	8.763	0.500	0.560	0.358	0.361
4023	Alt. isoform/cryptic acceptor	ctacccttagCCTGGACACC	6.193	0.514	0.626	0.365	0.416
4136	Alt. isoform/cryptic donor	TGACCTACAAGtacgtgctg	9.678	0.500	0.596	0.323	0.459
4211	Alt. isoform/cryptic donor	GCATTCTGATgtacgtgaag	7.123	0.514	0.847	0.112	0.868
4230	Alt. isoform/cryptic donor	GCTGAAGAAAgtgtgcatcc	6.203	0.529	0.934	0.048	0.949
4266	Alt. isoform/cryptic acceptor	tgaaccttagGAAGGCTCAG	7.185	0.457	0.849	0.138	0.837
4306	Alt. isoform/cryptic donor	ACCGCTATTGgtctgctgac	5.056	0.529	0.865	0.097	0.888
4344	Alt. isoform/cryptic donor	CACTTACCCGgtgtctatga	6.061	0.514	0.900	0.077	0.914
4366	Alt. isoform/cryptic donor	TGGCTGCAGGgtaacctgaa	6.041	0.500	0.750	0.183	0.755
4414	Alt. isoform/cryptic acceptor	cgctatgaagGCTTACCACA	3.152	0.514	0.945	0.052	0.945
4459	Alt. isoform/cryptic acceptor	cgactttagACCCGGTGGA	3.313	0.500	0.787	0.200	0.746
4510	Constitutive donor	ATCGCTTCAGgtgaggttga	11.745	0.500	0.262	0.665	0.607
4511	Constitutive acceptor	atcgcttagGTGAGGTGA	6.293	0.529	0.441	0.541	0.185
4701	Constitutive acceptor	ttactgcagGCATCTTGCT	8.159	0.514	0.411	0.576	0.288
4773	Alt. isoform/cryptic donor	GTCTATCCACgtgacctgt	6.070	0.529	0.520	0.403	0.224
4809	Alt. isoform/cryptic donor	CCCTAATGAGgtgatccctt	6.780	0.529	0.856	0.106	0.877
4873	Alt. isoform/cryptic donor	ATGCTGCCTGgtgaggtgaa	9.597	0.529	0.762	0.188	0.753
4878	Alt. isoform/cryptic donor	GCCTGGTGAGgtgaacgaca	5.312	0.500	0.940	0.044	0.953
5001	Alt. isoform/cryptic acceptor	tgggccccagCAGCAACGAC	4.531	0.529	0.765	0.227	0.703
5004	Alt. isoform/cryptic acceptor	gccccagcagCAACGACCAG	3.016	0.529	0.837	0.157	0.813
5067	Alt. isoform/cryptic donor	CCTGGAAGAGgtgaaggact	8.199	0.514	0.764	0.180	0.764
5104	Alt. isoform/cryptic acceptor	ttacctgcagATGAGGTTC	11.438	0.514	0.591	0.398	0.325
5140	Alt. isoform/cryptic donor	TACGAGAAGGgtactctgga	5.097	0.471	0.916	0.059	0.936
5263	Alt. isoform/cryptic acceptor	gctgaccagATCATCAAGC	2.350	0.557	0.593	0.388	0.345
5416	Alt. isoform/cryptic donor	ATCCTCGAGGgtgatgagct	5.258	0.471	0.829	0.126	0.848
5542	Alt. isoform/cryptic acceptor	gctgtctcagCTGTTTCATGT	8.742	0.500	0.683	0.304	0.555
5549	Alt. isoform/cryptic donor	AGCTGTTCATgtacacctct	5.135	0.514	0.868	0.098	0.887
5569	Alt. isoform/cryptic acceptor	tccgtctaagAGGAACCAGC	3.653	0.514	0.927	0.070	0.924
5616	Alt. isoform/cryptic acceptor	ctcttgatagGGTGTGAGA	3.796	0.486	0.802	0.185	0.769
5616	Alt. isoform/cryptic donor	TCTTGATAGGgtgttgagat	4.789	0.457	0.911	0.062	0.931
5668	Alt. isoform/cryptic acceptor	taccgttaagATGACCTATG	4.339	0.500	0.796	0.196	0.754

5730	Alt. isoform/cryptic acceptor	atctcgacagCTACTGCAGC	3.975	0.414	0.845	0.148	0.825
5829	Alt. isoform/cryptic acceptor	gcgagtctagGAAGCGGGAC	4.419	0.486	0.590	0.389	0.340
5863	Alt. isoform/cryptic acceptor	tgggtccagACTGAGAAGT	7.317	0.543	0.600	0.390	0.350
5921	Alt. isoform/cryptic donor	TGCATGCTGTgtacgggtct	4.883	0.500	0.957	0.031	0.968
6139	Alt. isoform/cryptic acceptor	cgctgctcagAACAGGCTCC	2.442	0.529	0.816	0.176	0.784
6199	Alt. isoform/cryptic acceptor	ctactccaagTTCAATCTTG	4.578	0.529	0.930	0.066	0.929
6213	Alt. isoform/cryptic acceptor	atcttggcagGGGCTTCATC	4.671	0.500	0.678	0.310	0.543
6258	Alt. isoform/cryptic acceptor	ccatctacagCAAAGAGGAA	4.973	0.500	0.822	0.171	0.793
6337	Alt. isoform/cryptic acceptor	ctctgcacagCAGGATATGA	3.577	0.529	0.610	0.372	0.390
6340	Alt. isoform/cryptic acceptor	tgccacagcagGATATGAACC	5.307	0.543	0.575	0.395	0.313
6420	Alt. isoform/cryptic acceptor	ccatcaccagAGAGGACATC	2.479	0.514	0.962	0.036	0.963
6445	Constitutive acceptor	cattctcagAACGTGTGCC	9.539	0.500	0.209	0.784	0.733
6624	Constitutive acceptor	gtctctatagGACCCTGGAT	10.048	0.500	0.176	0.817	0.784
6654	Alt. isoform/cryptic acceptor	gttacatcagGCGGTCCGAT	3.376	0.500	0.765	0.221	0.712
6663	Alt. isoform/cryptic donor	GCGGTCCGATgtgagatacg	6.227	0.486	0.936	0.047	0.950
6722	Alt. isoform/cryptic donor	GCGGAACCATgtacaagatt	5.221	0.514	0.960	0.028	0.971
6763	Alt. isoform/cryptic acceptor	ctacgtgcagTTGATCGCAT	5.764	0.529	0.844	0.148	0.824
6801	Constitutive acceptor	tgtctcttagGACCCCTTTC	5.629	0.514	0.255	0.731	0.652
6850	Alt. isoform/cryptic donor	GACACCTACCgtgagtcct	8.138	0.443	0.627	0.307	0.511
6894	Alt. isoform/cryptic donor	GTTGACAAGgtcaacatta	6.897	0.471	0.855	0.108	0.874
6895	Alt. isoform/cryptic acceptor	gttcgacaagGTCAACATTA	2.380	0.486	0.870	0.126	0.855
6943	Alt. isoform/cryptic donor	CTCGAGCCAGgtgatgcttg	8.185	0.486	0.833	0.125	0.850
6944	Alt. isoform/cryptic acceptor	ctcgagccagGTGATGCTTG	5.597	0.457	0.761	0.232	0.696
6960	Constitutive acceptor	cttgcatagGATGACCACC	2.622	0.471	0.452	0.510	0.115
6990	unclassified donor	GATCGTCAAGgttaacgcca	6.349	0.471	0.500	0.414	0.000
7038	Alt. isoform/cryptic donor	TAAGCTGGTgtgaagatta	5.500	0.471	0.942	0.041	0.957
7071	Alt. isoform/cryptic donor	GAACCTCCGACgtgtgggaca	4.732	0.543	0.918	0.059	0.936
7124	Alt. isoform/cryptic acceptor	cttctgaagTTGGTGAGTG	3.877	0.543	0.743	0.247	0.667
7126	Alt. isoform/cryptic donor	CCTGAAGTTGgtgagtgtt	12.153	0.500	0.727	0.210	0.711
7242	Alt. isoform/cryptic acceptor	gcaacctcagCCAGCAGATC	3.540	0.500	0.601	0.379	0.370
7355	Alt. isoform/cryptic donor	GCGTGGAAGAgtagactec	4.790	0.514	0.943	0.040	0.957
7464	Alt. isoform/cryptic acceptor	cactgttcagCGAGATCGTG	6.043	0.529	0.637	0.349	0.453
7535	Alt. isoform/cryptic donor	TGATGCTGAAGtagtctctg	4.763	0.529	0.907	0.069	0.924



7585	Alt. isoform/cryptic donor	AGAGCTACTGgtatgcacgc	9.638	0.500	0.752	0.188	0.750
7648	Alt. isoform/cryptic donor	AACCTGCTGGgtatgattca	7.568	0.500	0.698	0.229	0.672
7722	Alt. isoform/cryptic acceptor	ccagccttagGAATGTGCTG	3.055	0.500	0.807	0.185	0.771
7758	Alt. isoform/cryptic acceptor	cttcgagcagGCGGATCAGG	6.039	0.514	0.607	0.379	0.375
7792	Alt. isoform/cryptic acceptor	ggatctgcagAACAACTTA	3.876	0.514	0.696	0.289	0.586
7922	Alt. isoform/cryptic donor	AGATGGACAGgtccgatgaa	6.684	0.514	0.941	0.042	0.955
7968	Constitutive acceptor	atgtgcttagGTTGGACGAG	2.272	0.529	0.343	0.637	0.461
8169	Alt. isoform/cryptic acceptor	ctaccgacagGCTTGTTAGG	8.327	0.529	0.960	0.038	0.961
8173	Alt. isoform/cryptic donor	GACAGGCTTGtagctctc	8.257	0.486	0.774	0.169	0.782
8205	Alt. isoform/cryptic donor	TGCTCTTAAGgtgtaccca	6.332	0.514	0.932	0.049	0.948
8206	Alt. isoform/cryptic acceptor	tgtcttaagGTGTACGCCA	5.395	0.514	0.524	0.455	0.133
8331	Alt. isoform/cryptic acceptor	tgagcggcagGGTGAAGAAG	5.008	0.514	0.856	0.139	0.838
8353	Alt. isoform/cryptic acceptor	tcttattcagTTAGGGACG	2.472	0.500	0.856	0.133	0.845
8418	Alt. isoform/cryptic acceptor	acttcttagCAGGCACCTT	2.679	0.500	0.944	0.052	0.945
8421	Alt. isoform/cryptic acceptor	tccttagcagGCACCTCTT	6.406	0.514	0.782	0.208	0.734
8459	Alt. isoform/cryptic donor	TGTACGGCCGgtacacctac	4.997	0.471	0.903	0.074	0.918
8472	Alt. isoform/cryptic acceptor	acacctacagCGATATCAAC	3.154	0.543	0.614	0.363	0.409
8497	Alt. isoform/cryptic acceptor	catcatgcagACCCGGGAAA	2.271	0.471	0.944	0.053	0.944
8521	Constitutive acceptor	cctctctaagATCTCCGAGT	5.017	0.500	0.424	0.562	0.245
8538	Alt. isoform/cryptic donor	GTTGGATGAGgtggtgaaa	4.678	0.529	0.945	0.039	0.958
8583	Constitutive acceptor	cttaccttagGGGCGAAGAG	8.039	0.486	0.196	0.792	0.753

The putative intron splicing sites of wild-type RdRp gene sequence was predicted by Alternative Splice Site

Predictor (ASSP) (<http://wangcomputing.com/assp/>).

\* Scores of the preprocessing models reflecting splice site strength, i.e. a PSSM for putative acceptor sites, and an MDD model for putative donor sites. Intron GC values correspond to 70 nt of the neighboring intron.

\*\* Activations are output values of the backpropagation networks used for classification. High values for one class with low values of the other class imply a good classification. Confidence is a simple measure expressing the differences between output activations. Confidence ranges between zero (undecided) to one (perfect classification).



**Table S2. The predicted intron splicing sites of wild-type GP gene.**

Position (bp)	Putative splice site	Sequence	Score*	Intron GC*	Activations**		
					Alt./Cryptic	Constitutive	Confidence**
36	unclassified donor	GGTGGTGAAGgtgagcctgt	13.078	0.514	0.408	0.496	0.000
93	Constitutive acceptor	ttatttcagGGCTACCGAC	11.782	0.500	0.182	0.810	0.775
183	Alt. isoform/cryptic acceptor	ccgctgetagCATTCAGCGG	3.056	0.500	0.929	0.067	0.928
259	Alt. isoform/cryptic donor	AGGCAGATTCgtgaggaaga	4.761	0.500	0.860	0.108	0.875
297	Alt. isoform/cryptic acceptor	tcgctggcagCACTACCCAG	5.498	0.514	0.952	0.046	0.952
318	Alt. isoform/cryptic donor	GATTATCTCCgttagcgacc	4.792	0.529	0.928	0.056	0.940
324	Alt. isoform/cryptic acceptor	tctccgttagCGACCTGCCT	2.244	0.471	0.679	0.308	0.547
364	Alt. isoform/cryptic acceptor	atctctgaagTGCAGAGATCA	3.458	0.500	0.845	0.145	0.828
405	Alt. isoform/cryptic donor	TTACTACCAGgtcgagaaca	5.199	0.529	0.954	0.034	0.965
406	Alt. isoform/cryptic acceptor	ttactaccagGTCGAGAACA	5.238	0.500	0.592	0.383	0.354
437	Alt. isoform/cryptic donor	ACTCTTGCGTgtcagattct	4.710	0.529	0.923	0.057	0.938
443	Alt. isoform/cryptic acceptor	tgcgtgtcagATTCTGCTGA	5.120	0.514	0.630	0.357	0.433
504	Alt. isoform/cryptic donor	GTTCTCTAAGgtgccagtga	9.430	0.500	0.831	0.124	0.851
529	Alt. isoform/cryptic acceptor	tatcaccaagCTGGACAACA	2.328	0.500	0.853	0.139	0.837
552	Alt. isoform/cryptic donor	GCACTTCTCTgtggcacca	6.302	0.514	0.893	0.079	0.912
592	Constitutive acceptor	tctgaccagGACAACCTACC	3.778	0.543	0.341	0.630	0.460
649	Alt. isoform/cryptic acceptor	gtctcttcagACCGTTAAGC	6.880	0.529	0.693	0.295	0.574
708	Alt. isoform/cryptic donor	CCCGTATACCGtgagcatta	8.383	0.514	0.655	0.278	0.576
728	Constitutive acceptor	acctctccagAGAAGATCAT	6.756	0.514	0.388	0.598	0.352
780	Alt. isoform/cryptic donor	TGAGCACAAGgtgatctcat	9.170	0.514	0.526	0.387	0.264
824	Alt. isoform/cryptic acceptor	ttaccgaagAGATGCTGGA	3.783	0.529	0.805	0.185	0.770
835	Alt. isoform/cryptic donor	ATGCTGGATGgtgagcaca	9.886	0.514	0.525	0.389	0.260
894	Alt. isoform/cryptic donor	CAACAAGCGTgtgaggact	8.781	0.500	0.825	0.132	0.840
914	Alt. isoform/cryptic donor	GCATCATCAAgtagcagaag	6.287	0.514	0.880	0.089	0.899
969	Constitutive acceptor	cttgattagGCTGATCCTG	5.033	0.486	0.190	0.798	0.761
1008	Constitutive acceptor	tcctattagATGGCTGGTG	7.158	0.500	0.176	0.808	0.782
1046	Alt. isoform/cryptic donor	TTTTCTGTGgtacgatctg	8.598	0.486	0.789	0.158	0.800
1106	Alt. isoform/cryptic donor	GCCTGTGGAAGtagcttccg	5.205	0.500	0.934	0.049	0.948
1123	Alt. isoform/cryptic acceptor	tcggtcaagTGCAGCAACT	4.432	0.471	0.799	0.192	0.760
1128	Alt. isoform/cryptic acceptor	tcaagtcagCAACTGCGGC	3.238	0.486	0.858	0.136	0.842

1328	Alt. isoform/cryptic donor	GCCTGCTTAAGttcgtgacc	5.154	0.514	0.929	0.051	0.945
1356	Alt. isoform/cryptic donor	GATCGGTCTGgtgatcetta	4.853	0.486	0.642	0.284	0.558
1368	Alt. isoform/cryptic acceptor	tgatccttagCCAGATGCCT	2.891	0.500	0.795	0.198	0.751
1372	Alt. isoform/cryptic acceptor	ccttagccagATGCCTATGT	3.041	0.529	0.730	0.259	0.646
1443	Alt. isoform/cryptic donor	CCCTGTGCTGgtgaccteta	6.777	0.471	0.866	0.100	0.884
1463	Alt. isoform/cryptic donor	AGTTTGAAAAgtgccctgag	5.015	0.543	0.964	0.026	0.973
1631	Alt. isoform/cryptic donor	TGGGCTGCGAgtaacctggac	4.582	0.500	0.967	0.024	0.975
1783	Alt. isoform/cryptic donor	AAGATCAGCGgtatgatgc	7.260	0.529	0.671	0.262	0.610
1795	Alt. isoform/cryptic donor	ATGATCGCTGgtgacagcct	7.023	0.500	0.803	0.149	0.814
1883	Alt. isoform/cryptic donor	TGGATGGCAAgtaaccgttat	5.025	0.500	0.926	0.053	0.943
1889	Alt. isoform/cryptic donor	GCAAGTACCGgtatatggtt	5.618	0.486	0.814	0.135	0.834
1992	Alt. isoform/cryptic donor	CATCAAGAGCGtgggcatcc	5.600	0.514	0.825	0.138	0.833
2010	Alt. isoform/cryptic donor	CCACTACGAGgtgtcagaga	7.706	0.500	0.765	0.178	0.767
2044	Alt. isoform/cryptic acceptor	tcctattcagTCTACCCACA	4.199	0.514	0.801	0.186	0.768
2077	Constitutive donor	ACCTGCACCGgtaattcgga	7.342	0.500	0.414	0.502	0.176
2097	Alt. isoform/cryptic acceptor	atacctgcagAAAGAACCAG	4.833	0.529	0.869	0.122	0.860
2125	Constitutive acceptor	aggetttcagGACTTCTGCA	3.662	0.500	0.451	0.529	0.148
2191	Alt. isoform/cryptic donor	ATTAACGAGGgtgtacttg	4.511	0.529	0.955	0.033	0.966
2253	Alt. isoform/cryptic acceptor	ggatctacagCGTGCTGAAG	3.191	0.514	0.910	0.086	0.906
2280	Alt. isoform/cryptic donor	CGTTGCCGACgtgtgcatct	6.880	0.514	0.904	0.073	0.919
2331	Alt. isoform/cryptic donor	TACCGAAGAGgttccatacg	4.991	0.500	0.959	0.029	0.970
2359	Constitutive acceptor	tctgttcagGCTGATATTC	5.600	0.514	0.373	0.611	0.389
2401	Alt. isoform/cryptic donor	ATCACCATTGgtgagettat	11.534	0.529	0.522	0.395	0.242
2443	Alt. isoform/cryptic donor	ATCTACTCCGgtaacattgc	9.301	0.529	0.591	0.337	0.430
2469	Alt. isoform/cryptic donor	GAACGACCCGgtgaagatgt	5.862	0.500	0.788	0.167	0.788
2494	Alt. isoform/cryptic acceptor	tcactctcagCTTACTCATG	6.466	0.514	0.705	0.287	0.594
2618	Alt. isoform/cryptic donor	ACACTTACAGgttcaggctct	7.293	0.500	0.904	0.068	0.925
2619	Alt. isoform/cryptic acceptor	acacttacagGTTCAAGTCT	5.949	0.500	0.773	0.215	0.722
2624	Alt. isoform/cryptic donor	ACAGGTTACAGgtctgtctt	8.772	0.486	0.925	0.054	0.942
2625	Alt. isoform/cryptic acceptor	acaggttcagGTCTGGTCTT	3.359	0.514	0.684	0.296	0.567
2641	Alt. isoform/cryptic acceptor	tcttgagcagATCTCTGACA	4.449	0.529	0.945	0.051	0.946
2661	Alt. isoform/cryptic acceptor	tccecatcagCTTCAAGGAC	3.473	0.514	0.769	0.222	0.711
2668	Alt. isoform/cryptic acceptor	cagettcaagGACTTCAGCT	3.339	0.514	0.785	0.206	0.737

2676	Alt. isoform/cryptic acceptor	aggacttcagCTCATTCTTC	2.357	0.500	0.795	0.197	0.753
2751	Alt. isoform/cryptic donor	TCTTTTCAAGgttgacaccta	5.168	0.529	0.935	0.047	0.950
2752	Alt. isoform/cryptic acceptor	tcttttaagGTTGCACCTA	3.558	0.500	0.699	0.281	0.598
2787	Constitutive acceptor	cttctactagGCTTAAGTGC	5.710	0.500	0.455	0.528	0.138
2815	Alt. isoform/cryptic donor	CTTCTTTGTGgtcaggcct	9.649	0.529	0.757	0.184	0.757
2821	Alt. isoform/cryptic acceptor	ttgtgtcagGGCCTTTCTT	6.984	0.529	0.502	0.483	0.039
2865	Alt. isoform/cryptic acceptor	tgaccttcagCACCGCCATC	4.796	0.529	0.876	0.117	0.867
2908	Alt. isoform/cryptic acceptor	tacctaccagCTGGCTGTGA	6.831	0.500	0.696	0.293	0.579
2933	Alt. isoform/cryptic donor	GCAGCAACAAgtacaacac	6.089	0.500	0.851	0.108	0.873
2958	Alt. isoform/cryptic acceptor	tgttctgcagCGCAAACCCG	4.988	0.500	0.635	0.350	0.448
3021	Alt. isoform/cryptic donor	TTCTGTTGAGgtgctcgtga	5.200	0.514	0.963	0.028	0.971
3146	Alt. isoform/cryptic donor	ATTACATCAAgtccecgctc	5.088	0.500	0.945	0.041	0.956
3171	Alt. isoform/cryptic acceptor	tcattgccagCTACTTCGGC	3.870	0.471	0.816	0.173	0.788
3183	Alt. isoform/cryptic acceptor	acttcggcagCTTCTTCGAT	3.003	0.486	0.868	0.128	0.853
3201	Alt. isoform/cryptic acceptor	atacaatcagGGTGATCCTG	2.559	0.471	0.787	0.200	0.745
3201	Alt. isoform/cryptic donor	TACAATCAGGgtgatcctgc	5.357	0.500	0.829	0.130	0.843
3249	Alt. isoform/cryptic acceptor	acttctgcagCATCCTGACC	7.746	0.471	0.516	0.472	0.086

The putative intron splicing sites of wild type GP gene sequence was predicted by ASSP).

\* Scores of the preprocessing models reflecting splice site strength, i.e. a PSSM for putative acceptor sites, and an MDD model for putative donor sites. Intron GC values correspond to 70 nt of the neighboring intron.

\*\* Activations are output values of the backpropagation networks used for classification. High values for one class with low values of the other class imply a good classification. Confidence is a simple measure expressing the differences between output activations. Confidence ranges between zero (undecided) to one (perfect classification).

**Table S3. List of primers used in the study.**

Construct	Abbreviation	Primer sequence (5' to 3')	Purpose
p2300-N	N	<b>F:</b> GGGGTACCATGTCTAAGGTTAAGCTC	To amplify TSWV N and cloned into p2300S
		A	
		<b>R:</b> ACGTCGACTTAAGCAAGTTCTGCAA GTTTGG	
p2300-RdRp <sup>wt</sup>	RdRp <sup>wt</sup>	<b>F:</b> CGGGATCCATGAACATCCAGAAAATA	To amplify TSWV wildtype RdRp and cloned into p2300S
		C	
		<b>R:</b> GACGTCGACTTAATCCGTGCTCTTCTT CTTC	
p2300-RdRp <sup>opt</sup>	RdRp <sup>opt</sup>	<b>F:</b> CTCGGTACCATGAACATTCAGAAGAT	To amplify TSWV optimized RdRp and cloned into p2300S
		CCAAAAGC	
		<b>R:</b> GACTCTAGACTAATCGGTGCTCTCTT CCTC	
pCXSN-NSs	NSs	<b>F:</b> CTCGGTACCATGTCTTCAAGTGTTA	To amplify TSWV NSs and cloned into pCXSN
		TGAG	
		<b>R:</b> GACTCTAGATTATTTGATCCTGAAG ATATG	
pCB301-HH-S <sub>(-)</sub> -RZ-NOS	S <sub>(-)</sub>	<b>F:</b> CGAAAACCCGGTATCCCGGGTTCAG	To amplify the TSWV genomic RNA sequence for construction of S <sub>(-)</sub>
		AGCAATTGTGCATAATTTTATTC	
		<b>R:</b> GGTGGAGATGCCATGCCGACCCAGA	
		GCAATTGTGTCAATTTTATTCAAAC	
		<b>F:</b> GTTTGAATAAAATTGACACAATTGCT	To amplify the pCB301 backbone for construction of S <sub>(-)</sub>
pCB301-HH-S <sub>(+)</sub> -RZ-NOS	S <sub>(+)</sub>	CTGGGTCGGCATGGCATCTCCACC	
		<b>R:</b> GAATAAAATTATGACACAATTGCTCT	
		GAACCCGGGATACCGGGTTTTCG	
		<b>F:</b> CGAAAACCCGGTATCCCGGGTTCAG	To amplify the TSWV antigenomic RNA sequence for construction of S <sub>(+)</sub>
		AGCAATTGTGTCATAATTTTATCAAAC	
pCB301-HH-S <sub>(-)</sub> eGFP-RZ-NOS	SR <sub>(-)</sub> eGFP	<b>R:</b> GGTGGAGATGCCATGCCGACCCAGA	
		GCAATTGTGTGCATAATTTTATCTTAA	To amplify the pCB301 backbone for construction of S <sub>(+)</sub>
		<b>F:</b> GAATAAAATTATGACACAATTGCTCT	
		GGGTCGGCATGGCATCTC	
		<b>R:</b> GTTTGAATAAAATTGACACAATTGCT	To amplify the pCB301 backbone for construction of S <sub>(+)</sub>
pCB301-HH-S <sub>(-)</sub> eGFP-RZ-NOS	SR <sub>(-)</sub> eGFP	CTGAACCCGGGATACCGGGTTTTCG	
		<b>F:</b> GCTTTTTTATAATTTAACTTACAAC	To amplify the eGFP for construction of SR <sub>(-)</sub> eGFP
		GCTTTTACTTGTACAGCTCGTCCATGCC	
		GAGA	
		<b>R:</b> GTCAAAGCATATAACAATTCTACG	To amplify the pCB301 backbone for construction of S <sub>(+)</sub>
pCB301-HH-S <sub>(-)</sub> eGFP-RZ-NOS	SR <sub>(-)</sub> eGFP	ATCATCATGGTGAGCAAGGGCGAGGAG	
		CTGTTC	
		<b>F:</b> GAACAGCTCCTCGCCCTTGCTCACC	
			To amplify the pCB301 backbone for construction of S <sub>(+)</sub>

		ATGATGATCGTAGAAGTTGTTATATGCT TTGAC <b>R:</b> TCTCGGCATGGACGAGCTGTACAAG TAAAAGCAGTTGTAAGTTAAATTATAAA AAAGC	construction of SR <sub>(-)eGFP</sub>
pCB301-HH-S <sub>(-)mCherry&amp;eGFP</sub> -RZ-NOS	SR <sub>(-)mCherry&amp;eGFP</sub>	<b>F:</b> CACAGTACCAATAACCATAATGGTGA GCAAGGGCGAGGAGGATAAC <b>R:</b> GAAAAGCTGGACACGGCAAGATTA AGATCTGTACAGCTCGTCCATGCCGC <b>F:</b> GCGGCATGGACGAGCTGTACAGATC TTAATCTTGCCGTGTCCAGCTTTTC <b>R:</b> GTTATCCTCCTCGCCCTTGCTCACCA TTATGGTTATTGGTACTGTG	To amplify the mCherry for construction of SR <sub>(-)mCherry&amp;eGFP</sub>  To amplify the pCB301 backbone for construction of SR <sub>(-)mCherry&amp;eGFP</sub>
pCB301-T7-S <sub>(-)mCherry&amp;eGFP</sub> -RZ-NOS	T7:SR <sub>(-)mCherry&amp;eGFP</sub>	<b>F:</b> GAAATTAATACGACTCACTATAGGAG AGCAATTGTGCAATTTTATTCAAAC <b>R:</b> GGTGGAGATGCCATGCCGACCCAGA GCAATTGTGTCATAATTTATTCTTA <b>F:</b> GTTTGAATAAAATTGACACAATTGCT CTCCTATAGTGAGTCGTATTAATTC <b>R:</b> GAATAAAATTATGACACAATTGCTCT GGGTCGGCATGGCATCTC	To amplify the eGFP and mCherry expression cassette for construction of T7:SR <sub>(-)mCherry&amp;eGFP</sub>  To amplify the pCB301 backbone for construction of T7:SR <sub>(-)mCherry&amp;eGFP</sub>
pCB301-HH-S <sub>(+)eGFP</sub> -RZ-NOS	SR <sub>(+)eGFP</sub>	<b>F:</b> GGAAAAGCTGGACACGGCAAGATTA CTTGACAGCTCGTCCATGCCGAG <b>R:</b> GAACACAGTACCAATAACCATAATG GTGAGCAAGGGCGAGGAGCTGTTC <b>F:</b> GAACAGCTCCTCGCCCTTGCTCACCA TTATGGTTATTGGTACTGTGTTC <b>R:</b> CTCGGCATGGACGAGCTGTACAAGT AATCTTGCCGTGTCCAGCTTTTCC	To amplify the eGFP for construction of SR <sub>(+)eGFP</sub>  To amplify the pCB301 backbone for construction of SR <sub>(+)eGFP</sub>
pCB301-HH-S <sub>(-)mCherry&amp;eGFP</sub> <sup>Δ5'UTR</sup> -RZ-NOS	SR <sub>(-)mCherry&amp;eGFP</sub> <sup>Δ5'UTR</sup>	<b>F:</b> CGAAAACCCGGTATCCCGGGTTCAT GGTGAGCAAGGGCGAGGAGGATAAC <b>R:</b> GGTGGAGATGCCATGCCGACCCAGA GCAATTGTGTCATAATTTATTCAAAC <b>F:</b> GTTTGAATAAAATTGACACAATTGCT CTGGGTGCGCATGGCATCTCCACC <b>R:</b> GTTATCCTCCTCGCCCTTGCTCACCA TGAACCCGGGATACCGGTTTTCG	To amplify the Δ5'UTR expression cassette for construction of SR <sub>(-)mCherry&amp;eGFP</sub> <sup>Δ5'UTR</sup>  To amplify the pCB301 backbone for construction of SR <sub>(-)mCherry&amp;eGFP</sub> <sup>Δ5'UTR</sup>
pCB301-HH-S <sub>(-)mCherry&amp;eGFP</sub> <sup>ΔIGR</sup> -RZ-NOS	SR <sub>(-)mCherry&amp;eGFP</sub> <sup>ΔIGR</sup>	<b>F:</b> CGAAAACCCGGTATCCCGGGTTCAG AGCAATTGTGTCATAATTTATTTC <b>R:</b> GCATGGACGAGCTGTACAAGTAATT AAGATCTGTACAGCTCGTC <b>F:</b> GACGAGCTGTACAGATCTTAATTAC TTGTACAGCTCGTCCATGCCGAGA	To amplify the ΔIGR expression cassette for construction of SR <sub>(-)mCherry&amp;eGFP</sub> <sup>ΔIGR</sup>  To amplify the pCB301 backbone for construction of SR <sub>(-)mCherry&amp;eGFP</sub> <sup>ΔIGR</sup>

		<b>R:</b> GAATAAAATTATGACACAATTGCTCT GAACCCGGGATACCGGGTTTTCG	
pCB301-HH-S <sub>(-)mCherry&amp;eGFP</sub> <sup>Δ5'UTR</sup> -RZ-NOS	SR <sub>(-)mCherry&amp;eGFP</sub> <sup>Δ5'UTR</sup>	<b>F:</b> CGAAAACCCGGTATCCCGGGTTCAG AGCAATTGTGTCATAATTTIATTC <b>R:</b> GGTGGAGATGCCATGCCGACCCATG GTGAGCAAGGGCGAGGAGCTGTTC <b>F:</b> GAACAGCTCCTCGCCCTTGCTCACC ATGGGTCGGCATGGCATCTCCACC <b>R:</b> GAATAAAATTATGACACAATTGCTCT GAACCCGGGATACCGGGTTTTCG	To amplify the Δ3'UTR expression cassette for construction of SR <sub>(-)mCherry&amp;eGFP</sub> <sup>Δ5'UTR</sup>  To amplify the pCB301 backbone for construction of SR <sub>(-)mCherry&amp;eGFP</sub> <sup>Δ5'UTR</sup>
pCB301-HH-M <sub>(-)mCherry</sub> -RZ-NOS	M <sub>(-)</sub>	<b>F:</b> CGAAAACCCGGTATCCCGGGTTCAG AGCAATCAGTGCATCAGAAATATACC <b>R:</b> GGTGGAGATGCCATGCCGACCCAGA GCAATCAGTGCAAACAAAAAC <b>F:</b> GTTTTGTGTTGCACTGATTGCTCTGG GTCGGCATGGCATCTCCACC <b>R:</b> GGTATATTCTGATGCACTGATTGCT CTGAACCCGGGATACCGGGTTTTCG	To amplify the TSWV genomic M-RNA sequence for construction of M <sub>(-)</sub>  To amplify the pCB301 backbone for construction of M <sub>(-)</sub>
pCB301-HH-M <sub>(+)mCherry</sub> -RZ-NOS	M <sub>(+)</sub>	<b>F:</b> CGAAAACCCGGTATCCCGGGTTCAG AGCAATCAGTGCAAACAAAACTC <b>R:</b> GGTGGAGATGCCATGCCGACCCAGA GCAATCAGTGCGTCAGAAATATAC <b>F:</b> GTATATTCTGACGCACTGATTGCTC TGGGTCGGCATGGCATCTCCACC <b>R:</b> GAGTTTTTGTGTTGCACTGATTGCTCT GAACCCGGGATACCGGGTTTTCG	To amplify the TSWV antigenomic M-RNA sequence for construction of M <sub>(+)</sub>  To amplify the pCB301 backbone for construction of M <sub>(+)</sub>
pCB301-HH-M <sub>(-)eGFP</sub> -RZ-NOS	MR <sub>(-)eGFP</sub>	<b>F:</b> GAATCAAATTTAGCCTGTGACAAGC AGACTTACTTGTACAGCTCGTCCATGC <b>R:</b> CCATTATAATCTGAGCAGACGTATA AGATGGTGAGCAAGGGCGAGGAGCTG <b>F:</b> CAGCTCCTCGCCCTTGCTCACCATCT TATACGCTGCTCAGATTATAATGG <b>R:</b> GCATGGACGAGCTGTACAAGTAAGT CTGCTTGTCACAGGCTAAATTTGATTCTG	To amplify the eGFP for construction of MR <sub>(-)eGFP</sub>  To amplify the pCB301 backbone for construction of MR <sub>(-)eGFP</sub>
pCB301-HH-M <sub>(-)mCherry</sub> -RZ-NOS	MR <sub>(-)mCherry</sub>	<b>F:</b> GAATCAAATTTAGCCTGTGACAAGC AGACTTAAGATCTGTACAGCTCGTCCAT GC <b>R:</b> CCATTATAATCTGAGCAGACGTATA AGATGGTGAGCAAGGGCGAGGAGGAT AAC <b>F:</b> GTTATCCTCCTCGCCCTTGCTACCA TCTTATACGCTGCTCAGATTATAATGG <b>R:</b> GCATGGACGAGCTGTACAGATCTTA	To amplify the mCherry for construction of MR <sub>(-)mCherry</sub>  To amplify the pCB301 backbone for construction of MR <sub>(-)mCherry</sub>



		AGTCTGCTTGTCACAGGCTAAATTTGAT	
		TC	
pCB301-HH-M <sub>(-)GFP&amp;NSmMut</sub> -RZ-NOS	MR <sub>(-)GFP&amp;NSmMut</sub>	<b>F:</b> CTCTACCTTAGGCTGTGAACTCAA	
		AATGTAGACTCTTTTCGGTAATAAGG	To amplify the NSm <sup>Mut</sup> for construction
		<b>R:</b> GCATGGACGAGCTGTACAAGTAAGT	of MR <sub>(-)GFP&amp;NSmMut</sub>
		CTGCTTGTCACAGGCTAAATTTGATTC	
		<b>F:</b> GAATCAAATTTAGCCTGTGACAAGC	
		AGACTTACTTGTACAGCTCGTCCATGC	To amplify the pCB301 backbone for
pCB301-HH-L <sub>(-)GFP</sub> -RZ-NOS	L <sub>(-)GFP</sub>	<b>R:</b> CCTTATTACCGAAAAGAGTCTACAT	construction of MR <sub>(-)GFP&amp;NSmMut</sub>
		TTTGAGTTCAACAGCCTAAGGTAGAG	
		<b>F:</b> CGAAAACCCGGTATCCCGGGTTCAG	
		AGCAATCAGGTACAATAAAAC	To amplify the TSWV genomic L-RNA
		<b>R:</b> GGTGGAGATGCCATGCCGACCCAGA	for construction of L <sub>(-)GFP</sub>
		GCAATCAGGTAAACAACGAT	
pCB301-HH-L <sub>(-)GFP</sub> -RZ-NOS	L <sub>(-)GFP</sub>	<b>F:</b> ATCGTTGTACCTGATTGCTCTGGGT	
		CGGCATGGCATCTCCACC	To amplify the pCB301 backbone for
		<b>R:</b> GTTTTAGTTGTACCTGATTGCTCTGA	construction of L <sub>(-)GFP</sub>
		ACCCGGGATACCGGGTTTTTCG	
		<b>F:</b> CGAAAACCCGGTATCCCGGGTTCAG	
		AGCAATCAGGTAAACAACGAT	To amplify the TSWV antigenomic L-
pCB301-HH-L <sub>(+)GFP</sub> -RZ-NOS	L <sub>(+)GFP</sub>	<b>R:</b> GTGGAGATGCCATGCCGACCCAGAG	RNA for construction of L <sub>(+)GFP</sub>
		CAATCAGGTACAATAAAAC	
		<b>F:</b> GTTTTAGTTGTACCTGATTGCTCTGG	
		GTCGGCATGGCATCTCCACC	To amplify the pCB301 backbone for
		<b>R:</b> ATCGTTGTACCTGATTGCTCTGAAC	construction of L <sub>(+)GFP</sub>
		CCGGGATACCGGGTTTTTCG	
pCB301-HH-L <sub>(+)GFP</sub> -RZ-NOS	L <sub>(+)GFP</sub>	<b>F:</b> ATCAGGTAACAACGATTTTAAGCAA	
		ACATGAACATTGAGAAGATCCAAAAGC	To amplify the RdRp-optimized for
		TG	construction of L <sub>(+)GFP</sub>
		<b>R:</b> CATGCATTGTTAGGCATTACTTTTAA	
		TCTAATCGGTGCTCTCTCTCATCAG	
		<b>F:</b> CTGATGAGGAAGAGGACACCGATT	
pCB301-HH-M <sub>(-)GFP</sub> -RZ-NOS	M <sub>(-)GFP</sub>	AGATTAAAAGTAATGCCTAACAATGCA	To amplify the pCB301 backbone for
		TG	construction of L <sub>(+)GFP</sub>
		<b>R:</b> CAGCTTTTGGATCTTCTGAATGTTCA	
		TGTTTGCTTAAAATCGTTGTACCTGAT	
		<b>F:</b> ACCATTATAATCTGAGCAGACGTAT	
		AAGATGAGGATCCTGAAGCTTCTTG	To amplify the GP-optimized for
pCB301-HH-M <sub>(-)GFP</sub> -RZ-NOS	M <sub>(-)GFP</sub>	<b>R:</b> GAATCAAATTTAGCCTGTGACAAGC	construction of M <sub>(-)GFP</sub>
		AGACCTAAACAAGATGAGAGAAATC	
		<b>F:</b> GATTCTCTCATCTTGTAGTCTG	
		CTTGTCACAGGCTAAATTTGATTC	To amplify the pCB301 backbone for
		<b>R:</b> CAAGAAGCTTCAGGATCCTCATCTT	construction of M <sub>(-)GFP</sub>

ATACGTCTGCTCAGATTATAATGGT		
pGEM-NSs	-	<p><b>F:</b> GTTAATACTAACGGAGTGAAAC</p> <p><b>R:</b> GATTGAAATTTGGCTTGAAACAGTA</p> <p>C</p> <p>To amplify the sense-NSs for construction of pGEM-NSs to generate the DIG-tabled probes of S vRNA in Northern blotting</p>
pGEM-anti-NSs	-	<p><b>F:</b> GATTGAAATTTGGCTTGAAACAGTA</p> <p><b>R:</b> GTTAATACTAACGGAGTGAAAC</p> <p>To amplify the antisense-NSs for construction of pGEM-anti-NSs to generate the DIG-tabled probes of S cRNA in Northern blotting</p>
pGEM-NSm	-	<p><b>F:</b> GCTTTGACTAAAGCTATGGATAC</p> <p><b>R:</b> TCTTGTATTCTTGGCTGCACATC</p> <p>To amplify the sense-NSm for construction of pGEM-NSm to generate the DIG-tabled probes of M vRNA in Northern blotting</p>
pGEM-anti-NSm	-	<p><b>F:</b> TCTTGTATTCTTGGCTGCACATC</p> <p><b>R:</b> GCTTTGACTAAAGCTATGGATAC</p> <p>To amplify the antisense-NSm for construction of pGEM-anti-NSm to generate the DIG-tabled probes of M cRNA in Northern blotting</p>
pGEM-L 3'UTR	-	<p><b>F:</b> AGAGCAATCAGGTACAATAAAAC</p> <p><b>R:</b> AAGTAATGCCTAACAATGCATGA</p> <p>To amplify the L 3'UTR for construction of pGEM- L 3'UTR to generate the DIG-tabled probes of L vRNA in Northern blotting</p>
pGEM-anti-L 3'UTR	-	<p><b>F:</b> AAGTAATGCCTAACAATGCATGA</p> <p><b>R:</b> AGAGCAATCAGGTACAATAAAAC</p> <p>To amplify the antisense-L 3'UTR for construction of pGEM-anti-L 3'UTR to generate the DIG-tabled probes of L cRNA in Northern blotting</p>
pGEM-eGFP	-	<p><b>F:</b> ATGGTGAGCAAGGGCGAGGAGCTGTTC</p> <p><b>R:</b> ATGGTGAGCAAGGGCGAGGAGCTGTTC</p> <p>To amplify the sense-eGFP for construction of pGEM-eGFP to generate the DIG-tabled probes of antisense-eGFP RNA in Northern blotting</p>
pGEM-anti-eGFP	-	<p><b>F:</b> ATGGTGAGCAAGGGCGAGGAGCTGTTC</p> <p><b>R:</b> ATGGTGAGCAAGGGCGAGGAGCTGTTC</p> <p>To amplify the antisense-eGFP for construction of pGEM-anti-eGFP to generate the DIG-tabled probes of sense-eGFP RNA in Northern blotting</p>
-	-	<p><b>F:</b> GGTGGAGATGCCATGCCGACCCAGA</p> <p>GCAATTGTGTCATAATTTTATTCTTA</p> <p><b>R:</b> GGTGGAGATGCCATGCCGACCCAGA</p> <p>GCAATTGTGTCATAATTTTATTCAAAC</p> <p>To amplify the of S<sub>(+)eGFP</sub> minigenome by RT-PCR</p>
-	-	<p><b>F:</b> GTTCATTTCAATTGGAGAGGAGAGC</p> <p>ATCAGTGCAAAACAAAAAC</p> <p><b>R:</b> GGTGGAGATGCCATGCCGACCCAGA</p> <p>GCAATCAGTGCGTCAGAAATATAC</p> <p>To amplify the of M<sub>(-)mCherry</sub> and M<sub>(-)eGFP</sub> minigenome by RT-PCR</p>
-	-	<p><b>F:</b> GAATCAAATTTAGCCTGTGACAAGC</p> <p>To amplify the of M<sub>(-)opt</sub> genome by RT-</p>

	AGACCTAAACAAGATGAGAGAAATC	PCR
	<b>R:</b> GGTGGAGATGCCATGCCGACCCAGA	
	GCAATCAGTGCAAACAAAAAC	
	<b>F:</b> GATCAAGGATGTTAATTCAGCATGC	
	TTATCCCGATCCTCGAC	To amplify the of L <sub>(+)opt</sub> antigenome by
-	<b>R:</b> GAATCAAATTTAGCCTGTGACAAGC	RT-PCR
	AGACCTAAACAAGATGAGAGAAATC	