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Electronic Supplementary Material

Development of RNA Polymerase III-Driven Reverse Genetics System for the Rescue of a Plant Rhabdovirus

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Supplementary Materials and methods

Plasmid construction

To generate the plasmid p35S-MR for the transcription of SYNV genome-sense MR driven by the 35S promoter, we amplified the MR cassette from the previously described antigenome-sense MR construct pSYNV-MR$_{eGFP\text{-}DsRed}$ (Ganesan *et al*. 2013) with the primers Trailer/35S/F (5'-tttcatttggagaggAGAGACAAAAGCTCAGAACAATC-3') and Leader/HDV/R (5'-atgccatgccgacccAGAGACAGAAACTCAGAAAATAC-3'). The PCR products were ligated into the *Stu* I and *Sma* I doubled digested pCB301 binary plasmid (Yao *et al*. 2011), facilitated by the homologous sequences shown in lower case letters by ClonExpress II One Step Cloning (Vazyme, China).

The promoter sequences of *Arabidopsis thaliana U3B* (At5G53902), *U6* (At3G13855), and *tRNA^{Gly}-snoR43.1* (At1G06880) genes, were individually amplified by PCR from genomic DNA using the primer pairs U3B/F (5'-tgattacgccaagcttTTTACTTTAAATTTTTCTTATGGCT-3') and U3B/R (5'-

tgagcttttgtctctGACCAATGGTGCTCCCT-3'), U6/F (5'-tgattacgccaagcttCATTCGGAGtTTTTGTATCTTG-3') and U6/R (5'-tgagcttttgtctctCAATCACTACTTCGACTCTAGCTG-3'), and $tRNA^{Gly}$/F (5'-gggtggttttaagcttACTCATTCTAGCTTTCTTAC-3') and $tRNA^{Gly}$/R (5'-tgagcttttgtctctTGCACCAGCCGGGAATCG-3'), respectively. These promoter regions correspond to the nucleotide coordinates -325/+1 (*U3B*), -293/+1 (*U6*), and -127/+77 (*$tRNA^{Gly}$-snoR43.1*), with +1 denotes the transcription initiation site. The putative terminator sequence of *$tRNA^{Gly}$-snoR43.1* was amplified with the primer pair (T)n/F (5'-ggatggctaagggagTATTTTTAATTTTTATTTTTGAAATATTGCATTTTAAG-3') and (T)n/R (5'-acgacaatctgaattTAATTGGCAGTAGCTGTCAAAC-3'). The sequences of these promoters and terminator are shown in Supplementary Table S1. To generate the Pol III promoter-driven MR expression vectors pU3B-MR, pU6-MR, and ptRNA-MR, we amplified the SYNV genome-sense MR and the ribozyme sequence from the plasmid p35S-MR using Trailer/F (5'-agagacaaaagctcagaacaatc-3') and HDV/R (5'-gagctctcccttagccatc-3'). This fragment, along the individual Pol III promoter fragments and the terminator fragment described above, were inserted into the *Hin*d III and *Eco*R I digested pCB301 vector with ClonExpress MultiS One Step Cloning Kit (Vazyme, China).

To generate the plasmid pU6-tRNA-MR, we amplified the U6 promoter using the forward primer U6/F and reverse primer U6-tRNA/R (5'-CACTGGTGCTTTGTTCAATCACTACTTCGACTCTAGCT-3'). The fragment containing the $tRNA^{Gly}$, SYNV MR, and HDV sequence was amplified from the ptRNA-MR with the primers tRNA-MR/F (5'-AACAAAGCACCAGTGGTC-3') and (T)n/R. These two fragments were ligated into the *Hin*d III and *Eco*R I digested pCB301 vector with the aid of ClonExpress MultiS One Step Cloning reagent.

To construct the plasmids ptRNA-SYNV and p35S-SYNV for transcription of the full-length SYNV gRNA, we amplified the cDNA fragment from the plasmid pSYNV-GFP (Wang *et al*. 2015). The fragment was digested with *Fse* I and *Aat* II, and then inserted into the ptRNA-MR or p35S-MR plasmids digested by *Fse* I and *Aat* II.

Agroinfiltration

The above plasmid constructs were transformed into *Agrobacterium tumefaciens* strain EHA105 by electroporation. Agrobacterial cultures were resuspended in the buffer (10 mmol/L $MgCl_2$, 10 mmol/L MES, pH5.6, 100 μmol/L acetosyringone) and adjusted to an optical density at 600 nm (OD_{600}) of 0.7. SYNV minireplicon and agroinfection assays were carried out by adding one volume of the cell cultures harboring the MR or gRNA transcription plasmid to two volumes of 1:1 mixtures of *Agrobacterium* suspensions containing the plasmid pGD-NPL (Wang *et al.* 2015) and the viral suppressors of RNA silencing (VSRs) plasmids p19, γb, and HC-Pro (Qian *et al.* 2017). In the negative control experiments, we used the mixed pGD-N and pGD-P cultures (Ganesan *et al*. 2013) to replace the pGD-NPL strain. For determination of the transcript levels, the

Agrobacterium cultures harboring the plasmid pGD-NPL were omitted from the mixtures.

Fluorescence microscopy and photography

The fluorescent foci in infiltrated leaves were visualized with a Zeiss Lumar V12 epifluorescence microscope (Carl Zeiss, Germany). Filter set Lumar 38 (excitation, 470/40 nm; emission, 525/50 nm) and Lumar 31 (excitation, 565/30 nm; emission, 620/60 nm) were used for GFP and RFP detection. Infected plants were photographed with ultraviolet (UV) illumination.

Protein analysis

Total protein extracts separated by 12.5% SDS-PAGE were transferred to polyvinylidene difluoride (PVDF) membranes, followed by detection with polyclonal antisera specific to SYNV virions (Jackson and Christie, 1977) and *N. benthamiana* Actin (Sangon Biotech, Shanghai, China), or monoclonal antibodies against GFP and RFP (Abcam, Cambridge, UK).

Quantitative reverse transcription PCR (qRT-PCR)

Total RNA samples were isolated from leaf tissues infiltrated with the ptRNA-MR, p35S-MR, ptRNA-SYNV, and p35S-SYNV at 2 dpi and were reverse transcribed into cDNA using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Japan). qRT-PCR assays were performed by using SYBR Green I Master kit (Roche, Rotkreuz, Switzerland) with primers specific to *GFP*, SYNV *P* and *G* genes and *N. benthamiana Actin* gene. *N. benthamiana Actin* gene was used as an internal reference for the relative quantification.

Table S1 *Arabidopsis* Pol III promoters and terminator sequences used in this study

Name	Sequence (5'→3')
$tRNA^{Gly}$-snoR43.1 promoter	ACTCATTCTAGCTTTCTTACCAACTTGTCCCAATTCTTATTCAGTTATTCCATATCTTGACCAAAC CATTTTGATGAGAGTAAAAAAAAAGGTTTCTGGTATTTATTTTAAAACATAGCGTAAGGTTCAA CAAA**GCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGA TTCCCGGCTGGTGCA**
U3B promoter	TTTACTTTAAATTTTTCTTATGGCTCAGCCTGTGATGGATAACTGAATCAAACAAATGGCGTCTG GGTTTAAGAAGATCTGTTTTGGCTATGTTGGACGAAACAAGTGAACTTTTAGGATCAACTTCCGT TTATATACGGAGCTTATATCGAGCAATAAGATAAGTGGGCTTTTTATGTAATTTAATGGGCTATC GTCCATATATTCACTAATACCCATGCCCAGTACCCATGTATGCGTTTCATATAAGCTCCTAATTT CTCCCACATCGCTCAAATCTAAACAAATCTTGTTGTATATATAACACTGAGGGAGCACCATTGGT CA
U6 promoter	CATTCGGAGTTTTTGTATCTTGTTTCATAGTTTGTCCCAGGATTAGAATGATTAGGCATCGAACC TTCAAGAATTTGATTGAATAAAACATCTTCATTCTTAAGATATGAAGATAATCTTCAAAAGGCCC CTGGGAATCTGAAAGAAGAGAAGCAGGCCCATTTATATGGGAAAGAACAATAGTATTTCTTATA TAGGCCCATTTAAGTTGAAAACAATCTTCAAAAGTCCCACATCGCTTAGATAAGAAAACGAAGC TGAGTTTATATACAGCTAGAGTCGAAGTAGTGATTG
$tRNA^{Gly}$-snoR43.1 terminator	TATTTTTAATTTTTATTTTTGAAATATTGCATTTTAAGTTTATACAAACTATTAATATCTTGTATCT GCCAAAATAGCCTGCAAATTCCACAGAACCAATTATGTTCAAGAGCTTGAGAACAGAACGTTTA CAGGATTCTTCACATTAAATACTCAAAAAAGAAAGTTTACCCTTTTATGTTTGACAGCTACTGCC AATTA

Transcription initiation sites are underlined, and $tRNA^{Gly}$ sequence is marked in boldface.

References

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