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LETTER

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

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Dear Editor,

De novo generation of negative-stranded RNA viruses (NSVs) requires efficient transcription of integral viral RNAs with precise termini from cloned plasmids. Studies during the past 25 years with animal NSVs have established the bacteriophage T7 RNA polymerase (Pol)- and endogenous Pol I-based transcription systems as the most efficient platforms for recovery of recombinant NSVs (Bridgen 2012). Unfortunately, adaptation of these transcription systems to plants has thus far met with little success, and difficulties in delivery of multiple plasmids to plant cells further hamper the development of plant NSV reverse genetics systems (Jackson and Li 2016; Zang *et al.* 2020). Recently, several studies have reported strategies to overcome these technical barriers, resulting in recombinant recovery of Sonchus yellow net nucleorhabdovirus (SYNV) (Wang *et al.* 2015), barley yellow striate mosaic cytorhabdovirus (Gao *et al.* 2019), tomato spotted wilt tospovirus (TSWV) (Feng *et al.* 2020), and rose rosette emaravirus (Verchot *et al.* 2020). The procedures rely on agroinfiltration of *Nicotiana benthamiana* plants to express viral RNAs driven by a Pol II promoter, *i.e.*, cauliflower mosaic virus 35S promoter (p35S). Despite being successful, historical evidence from animal NSV reverse genetics studies indicates that the Pol II-driven systems may be limited in the range of NSVs that can be rescued (Bridgen 2012). Pol II transcripts are capped, polyadenylated and often spliced in the nucleus (Fong and Bentley 2001), which may affect the intergrity of viral RNAs. Indeed, the success of TSWV rescue lies in removal of the numerous cryptic intron-splicing sites in the viral genomes through codon optimization (Feng *et al.* 2020). Thus, establishment of alternative transcription systems suitable for plant NSV recovery is warranted.

Here by using SYNV as a model, we have exploited the utility of Pol III-driven system in NSV reverse genetics. In higher eukaryotes, Pol III initiates and terminates transcription at defined positions and is responsible for synthesis of 5S ribosomal (r)RNA, transfer (t)RNA, small nuclear (sn)RNAs and some non–protein-coding (nc)RNAs (Vannini and Cramer 2012). We first cloned the promoter of the *Arabidopsis thaliana* $tRNA^{Gly}$*-snoR43.1* gene (At1G06880), designated ptRNA (Supplementary Materials and Methods; Supplementary Table S1). The ptRNA was employed to drive the transcription of an SYNV genome-sense minireplicon (MR) cassette, which contained the green and red fluorescence protein (GFP and RFP) reporter genes flanked by the viral noncoding trailer and leader sequences (Fig. 1A). Transcription termination was dictated by the $tRNA^{Gly}$*-snoR43.1* terminator sequence consisting of a short run of "T" residues, and a precise MR gRNA 3′ terminus was generated by fusion with an autolytic hepatitis delta virus ribozyme (ΔRz). As the ptRNA promoter contains key *cis*-acting transcription elements within the transcribed tRNA sequence and initiates transcription internally, the resulting primary MR RNA transcripts would contain a 5′ $tRNA^{Gly}$ fusion. During tRNA maturation, the 3′ terminus of $tRNA^{Gly}$ is anticipated to be precisely cleaved by RNase Z (Kruszka *et al.* 2003). The tRNA cleavage should result in an accurate 5′ terminus of

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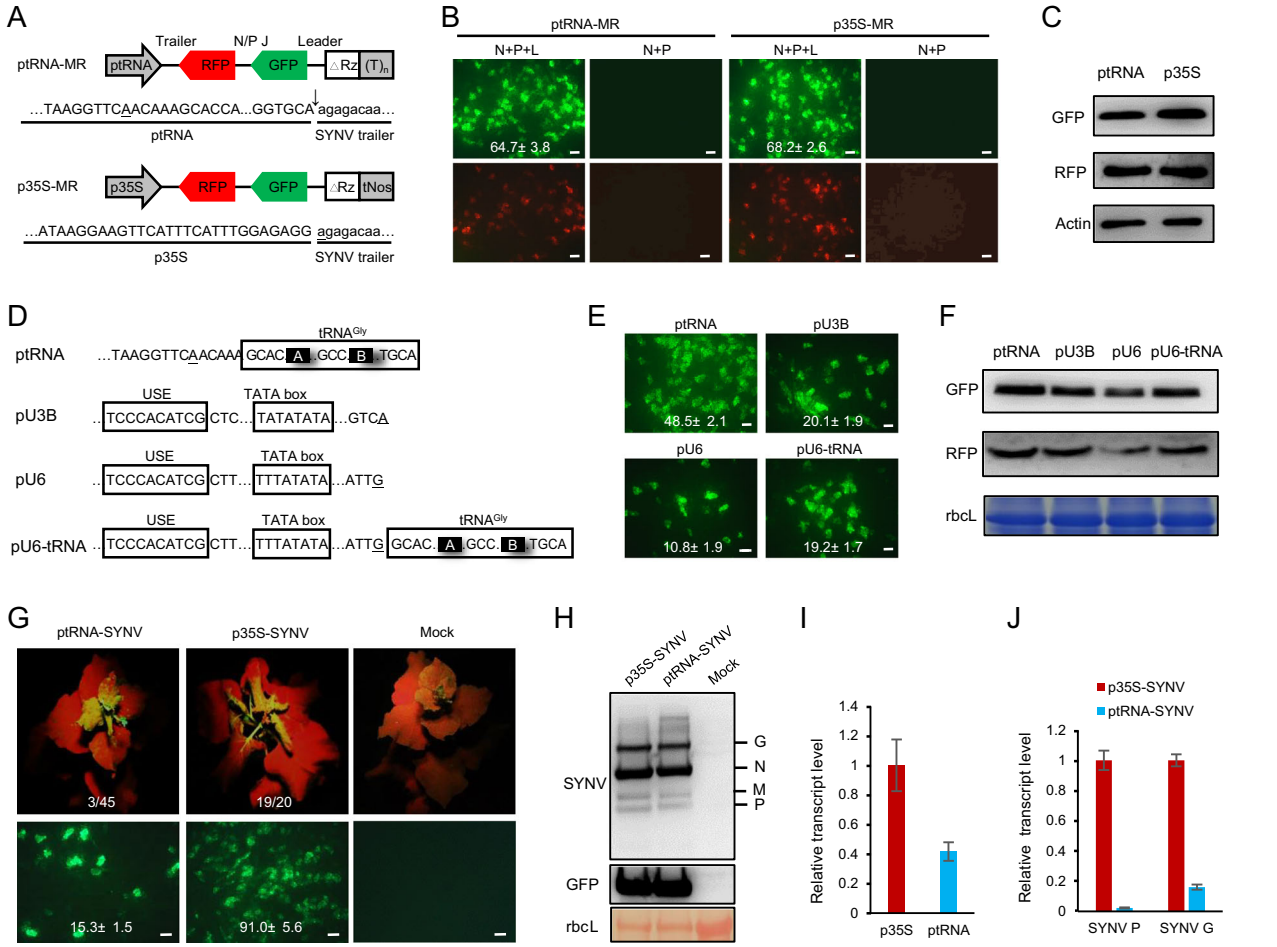

Fig. 1 Sonchus yellow net rhabdovirus (SYNV) reverse genetics systems based on an RNA polymerase (Pol) III promoter-driven transcription system. **A** Schematic representation of the SYNV minireplicon (MR) constructs. The vectors contain the SYNV MR cDNA flanked by either Pol III *Arabidopsis thaliana tRNA*^{Gly}*-snoR43.1* promoter (ptRNA) or Pol II cauliflower mosaic virus 35S promoter (p35S) and the hepatitis delta ribozyme (ΔRz). (T)n and tNos depict the terminators for Pol III and Pol II, respectively. Upper- and lower-case letters denote promoter and SYNV trailer sequences, and the tRNA sequence is highlighted in boldfaced letters. Vertical arrow depict the RNase Z processing site, and transcription initiation sites are underlined. N/P J, SYNV N/P gene junction. **B** Comparison of SYNV MR activity in ptRNA- and p35S-based reverse genetics systems. Leaf tissues of *Nicotiana benthamiana* were infiltrated with agrobacterial mixtures harboring plasmids for transcription of SYNV MR RNA, together with supporting plasmids for expression of SYNV core proteins and viral suppressors of RNA silencing. Agroinfiltrated leaf tissues were imaged at 8 days postinfiltration (dpi) with a fluorescence microscopy under GFP (upper panels) and RFP (lower panels) channels. The numbers of GFP-positive cells per observation field were counted. Data are presented as mean values ± SD based on six independent plants (n = 6). Scale bar = 200 μm. **C** Total protein extracted from infiltrated leaf tissues was analyzed by Western blotting with antibodies specific for GFP, RFP and Actin (an internal control). **D** Schematic representation of the organization and *cis*-elements of *A. thaliana* ptRNA, pU3B, and pU6 promoters. The tRNA gene body, TATA box, and upstream sequence element (USE) are labeled by open boxes, whereas the A- and B-box in the tRNA^{Gly} are denoted by black solid boxes. Transcription initiation sites are underlined. **E** GFP foci in *N. benthamiana* leaves infiltrated with agrobacterial mixtures harboring plasmids for transcription of SYNV MR RNA driven by four Pol III promoters, together with supporting plasmids for expression of SYNV core proteins and viral suppressors of RNA silencing. Images were taken at 8 dpi with a fluorescence microscopy under GFP channel. The numbers show the mean values ± SD of GFP-positive cells per field (n = 6). Scale bar = 200 μm. **F** The levels of GFP and RFP proteins in leaf tissues shown in (**E**) were analyzed by Western blotting with specific antibodies. The Coomassie Brilliant Blue-stained large subunit of RuBisCO (rbc L) serves as a protein loading control. **G** Recovery of recombinant SYNV harboring a GFP reporter using ptRNA- and p35S-driven rescue systems. Upper panels show infected and mock-infected plants imaged under ultraviolet light at 30 dpi. Number of infected per total agroinoculated plants pooled from three independent experiments is indicated. Bottom panels show GFP foci in infiltrated leaf tissues photographed at 9 dpi, with foci numbers presented as mean values ± SD from six plants. **H** Total protein samples extracted from systemically infected tissues were analyzed by Western blot using antibodies against SYNV virion and GFP. The Coomassie Brilliant Blue-stained large subunit of RuBisCO (rbc L) serves as a protein loading control. **I, J** qRT-PCR analyses of the relative transcript levels of SYNV MR RNA and gRNA. *N. benthamiana* leaves were agroinfiltrated to express the MR RNA (**I**) or full-length gRNA (**J**) driven by ptRNA or p35S. Note that RNA silencing suppressors were also co-expressed, but SYNV core proteins were omitted to avoid viral replication. Total RNA samples isolated from the infiltrated leaves at 2 dpi were analyzed by qRT-PCR with *GFP*- or SYNV *P*- and *G*-specific primers to quantify the levels of MR or gRNA transcripts, respectively. Data are presented as mean values ± SD based on three independent biological repeats (n = 3).

the flanking SYNV MR RNA (Fig. 1A). As a parallel control, we also constructed an analogous MR transcription plasmid driven by the 35S promoter (p35S-MR; Fig. 1A).

The ptRNA-MR and p35S-MR plasmids were individually delivered into *N. benthamiana* leaves through agroinfiltration, along with supporting binary plasmids designed for expression of the SYNV nucleoprotein (N), phosphoprotein (P), large RNA polymerase (L), and viral suppressors of RNA silencing, which are collectively required for MR rescue (Ganesan *et al.* 2013). At 8 days post infiltration (dpi), the infiltrated leaf tissues showed discrete cell foci expressing both GFP and RFP. Reporter gene expression from these MR constructs was dependent on viral replication, since we did not detected MR activity in control experiments where the L protein expression plasmid was omitted (Fig. 1B). In comparison, the ptRNA-MR and p35S-MR exhibited similar levels of reporter gene expression as revealed by fluorescence microscopy followed by quantitative analysis of GFP foci numbers (Fig. 1B) and protein gel blot analysis of GFP and RFP levels (Fig. 1C).

Unlike the ptRNA-like internal promoters, a minority of Pol III promoters, e.g., *A. thaliana* U3 and U6 RNA promoters, are located entirely upstream of the gene body and employ an upstream sequence element (USE) and the TATA box to recruit Pol III machinery (Orioli *et al.* 2012; Fig. 1D). To evaluate their utility in SYNV reverse genetics, we generated additional SYNV MR transcription plasmids driven by the *Arabidopsis* U3B (pU3B) and U6 (pU6) promoters (Sequence information please see Supplementary Table S1). pU3B-directed transcription initiates with "A" nucleotide, which is compatible with the first nucleotide of the SYNV MR RNA sequence. However, efficient transcription directed by pU6 prefers a "G" nucleotide, which would result in one additional nonviral "G" at the 5′ end of the MR transcripts. Thus, we also constructed a pU6-tRNA fusion promoter-driven MR transcription plasmid to generate the exact 5′ end of the SYNV MR RNA by utilizing the endogenous tRNA processing machinery (Fig. 1D). We next conducted MR assays through agroinfiltration as described above. All the four Pol III promoters supported SYNV MR rescue. By comparison, the highest levels of reporter gene expression were achieved with the MR driven by ptRNA, followed by pU3B and then pU6 (Fig. 1E, 1F). Notably, pU6-tRNA increased MR reporter expression to levels comparable to those supported by pU3B, suggesting that generation of precise viral 5′ end by removing the 5′ extra "G" nucleotide improved MR activity. Altogether, these results identify ptRNA as the most efficient promoter, which was chosen for subsequent studies.

We next investigated whether recombinant infectious SYNV could be rescued from cloned complementary (c)DNAs by using the Pol III transcription system. For this purpose, we replaced the MR cDNA in the ptRNA-MR plasmid with cDNA encoding a full-length SYNV gRNA derivatives containing a GFP expression unit inserted between the *N* and *P* genes to generate the ptRNA-SYNV plasmid. Note that SYNV gRNA rather than agRNA was expressed because our recent data showed that the gRNA strategy outperformed the initially developed agRNA strategy (Ma *et al.* 2020). Mixtures of agrobacterial suspensions harboring the ptRNA-SYNV and the necessary supporting binary plasmids were agroinfiltrated into lower leaves of *N. benthamiana* plants. Symptoms characteristics of SYNV infections started to appear on newly emerged upper leaves at 17 dpi, and viral infections were further confirmed by visualization of GFP reporter expression with UV illumination (Fig. 1G; upper panel). Ultimately, 3 out of 45 (6.7%) agroinfiltrated plants developed systemic infections. In parallel experiments conducted with the 35S promoter-driven expression of SYNV gRNA (p35S-SYNV), disease onset was observed as early as 15 dpi, and the recombinant virus was recovered from systemic leaf tissues in 19 out of 20 (95%) of agroinfiltrated plants. Nevertheless, the recombinant viruses recovered from both plasmids expressed similar levels of viral structure proteins and GFP protein in systemically infected leaf tissues (Fig. 1H). To further compare the rescue efficiency, we examined the fluorescent foci in the agroinfiltrated leaves that are indicative of primary virus rescue events. At 9 dpi, on average 15.3 GFP foci per field was observed in the ptRNA-SYNV infiltrated leaf tissues, which was about sixfold less than the 91 foci per field for the p35-SYNV tissues (Fig. 1G; bottom panel).

To ascertain the causes of the differential rescue efficiencies, we compared the levels of SYNV MR and gRNA transcripts produced by ptRNA and p35S in the absence of viral replication. Quantitative real-time reverse transcription PCR (qRT-PCR) assays showed that the MR transcripts produced by ptRNA reached 42% of those by p35S (Fig. 1I). In contrast, the gRNA transcripts generated by the ptRNA was only 2% or 16% of those made by p35S when analyzed with the SYNV *P*- or *G*- specific primers, respectively (Fig. 1J).

In nature, Pol III promoters are responsible for transcribing the small size 5S rRNA, tRNA, snRNA, and other small ncRNAs (Vannini and Cramer 2012). It is therefore probable that the Pol III enzyme is less processive than the Pol II machinery. Alternatively, premature termination may have occurred during Pol III transcription of SYNV gRNA, leading to low levels of integral transcripts. Transcription termination by Pol III is dictated by a simple, short run of T residues (T $\geq$ 4 in vertebrates and T $\geq$ 5 in yeast) (Orioli *et al.* 2012). The genome of rhabdoviruses contain such U-tracts in each of the gene junctions

(Jackson *et al.* 2005), which may have caused periodically Pol III transcription termination. Indeed, qRT-PCR assays using the 3′ proximal *P* gene-specific primers detected a much greater reduction of the SYNV gRNA transcripts than that by using the 5′ proximal *G* gene-specific primers. This difference may be explained by premature termination mediated by the three gene junction sequences between the *P* and *G* genes.

In conclusion, we have developed a Pol III-driven reverse genetics system for the rescue of a plant rhabdovirus. To our best knowledge, this is the first report of Pol III-based NSV reverse genetics systems. Future studies are warranted to explore the utility of the Pol III system for the rescue of other NSVs, particularly for those cytoplasmic viruses with splicing problems or without poly(U) tracts.

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Author Contributions ZL and XZ designed the study. XZ, KS, YL, and CZ performed the experiments. ZL and XZ analyzed and interpreted the data, and ZL and XZ wrote the manuscript. ZL revised and approved the final manuscript. All authors had full access to the final version of the report and agreed to the submission.

Compliance with Ethical Standards

Conflicts of interest The authors declare that they have no conflict of interest.

Animal and Human Rights This article does not contain any studies with human or animal subjects performed by any of the authors.

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