
SHORT COMMUNICATION

A New Viral Vector Exploiting RNA Polymerase I-Mediated Transcription

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Abstract—We have developed a new viral vector system exploiting RNA-polymerase I transcription. The vector is based on the crucifer-infecting tobacco mosaic virus (crTMV) cDNA inserted into the rRNA transcriptional cassette (promoter and terminator). To visualize reproduction of the vector, the coat protein gene was replaced with the gene encoding green fluorescent protein (GFP) resulting in a Pr^{rRNA}-crTMV-GFP construct. Our results showed that agroinjection of *Nicotiana benthamiana* leaves with this vector results in GFP production from uncapped crTMV-GFP RNA because RNA polymerase I mediates synthesis of rRNA lacking a cap. Coexpression of the crTMV 122 kDa capping protein gene and the silencing suppressor encoded by the *tomato bushy stunt virus p19* gene stimulated virus-directed GFP production more than 100-fold. We conclude that the Pol I promoter can be used to drive transcription in a transient expression system.

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Three different RNA polymerases (Pols), designated Pol I, II, and III, catalyze basic transcription [1]. All Pol II-transcribed RNAs have a 5'-cap structure that is important for pre-mRNA processing, mRNA export, translation, and quality control [2-4]. Pol I is involved in the high-level synthesis of the non-capped single-transcript rRNA precursor (pre-rRNA), which is processed into 28S (25S in plants), 5.8S, and 18S rRNAs [5-7].

Tobacco mosaic virus (TMV) replicates in the cytoplasm, and its genome is a single-stranded sense RNA (~6.5 kb) with an m⁷GpppG cap structure on its 5' end [8-10]. One of the TMV replicase components, the 126 kDa protein, performs the viral genomic RNA capping that is essential for recruiting translation initiation factors [11, 12] and for anchoring RNA to the actin/endoplasmic reticulum (ER) network [13]. Capping of viral RNA occurs through the guanylyltransferase activity of the vital 126 kDa capping enzyme [14, 15].

Agroinfection or *Agrobacterium*-delivered TMV vector insertion leads to virus infection of almost all leaf cells [16].

In the transient expression system, TMV-based viral vector is delivered to the cell nucleus with the help of *A. tumefaciens* T-DNA. Then, Pol II-synthesized viral capped genomic RNA is exported from the nucleus into the cytoplasm where it is translated to produce its own capping enzyme replicase complex. However, under natural conditions, plant RNA viruses replicate in the cytoplasm and are not adapted to the nuclear splicing machinery, which recognizes and removes cryptic introns from viral RNA, leading to the degradation of that RNA. Recently, to increase the level of infectious transcripts, the expensive method of TMV cDNA modification was developed. This method involves the removal of putative cryptic splice sites and the insertion of multiple plant introns [16].

We have created a system exploiting Pol I-directed transcription that does not require nuclear splicing. Previously, the Pol I^{rRNA} promoter was used in hybrid constructions for expressing noncoding shRNAs [17, 18] or negative-strand animal viral RNA molecules [19-24]. The Pol I (rRNA) promoter has an advantage in RNA production compared to Pol II. It was shown that Pol I-synthesized RNA molecules do not compete for nucleocytoplasmic export with Pol II-synthesized RNA [17]. This finding is important in cases of co-agroinjection of Pol I- and Pol II-based vectors.

Abbreviations: crTMV, crucifer-infecting TMV; IC, intermediate construct; IRES, internal ribosome entry site; TBSV, tomato bushy stunt virus; TMV, tobacco mosaic virus.

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nucleotides of the Pol I-promoter fused to the 68 bp 5' proximal to the crTMV genome (omega sequence) followed by IRES_{CP148} and the first 44 bp of the crTMV RdRp ORF was obtained by overlap PCR using the following primers: PolI-SphI-Dir1, PolI-Rev, 4351-MunI-Rev2, 4351-Dir2, Omega-IRES_R1 (GCTGCAACCGAATCGACGAATTGTTGTTGTTGTTGTGGTATTTTTG), IRES_D2 (AATTCGTCGATTCGGTGCAGCAT), IRES_R3 (ATTTTCTTCTTTCAAATAAACGAATC), and IRES_D4 (CGTTTAATTTGAAGAAGAAAATGGCACAAATTTCAAC). To obtain IC3, fragment 4 was digested with *SphI/MunI* and inserted into pSP72 containing fragment 2.

IC4. To obtain a fragment containing the *Saccharomyces cerevisiae* Pol I-terminator of transcription, the corresponding oligonucleotides were annealed pairwise, extended with Klenow fragment, and then amplified. The following yeast-specific overlapping primers were used: yT1-BHI-dir (TTTGATCCCTC-GAGGGTACCGCATGCAGATCTTAGA), yT1-rev (TAAAATTTATAGAGACTTGTTTCAGTCTAAGATCT-GCA), yT2-dir (CAAGTCTCTATAAATTTTATTTGT-CTTAAGAATTCTA), and yT2-rev (ACTGAGCTCTT-TTTACCCGGATCATAGAATTCTTAAG). This fragment 5, which was flanked by *Bam*HI/*Sac*I sites, was digested and inserted into pCambia1300 (Cambia, Australia) with a fragment of pICH4351 [27] flanked by *Pst*I/*Bam*HI sites and containing the 3' proximal part of the replicase, GFP, and the 3' UTR. The resulting plasmid was IC4.

In the final cloning step, fragments flanked by *HindIII*/*MunI* were obtained from IC1, IC2, and IC3. The construct crTMV-CP-GFP was digested with *MunI*/*PstI* to produce the fragment containing the part of the crTMV genome from 87 to 3670 nt. The fragments from IC1, IC2, and IC3 were ligated into IC4 digested with *HindIII*/*PstI*, resulting in the Pol I-crTMV-GFP viral vector (in the case of the IC1 fragment), Pol I^{rRNA}-5'IRES-crTMV-GFP (for the IC2 fragment), or Pol I^{rRNA}-IRES3'-crTMV-GFP (for the IC3 fragment). IC1* was used for the intron-containing vector, Pol I^{rRNA}-crTMV(intr)-GFP.

To obtain the Pol II^{Act2}-122 kDa construct, PCR was performed with the primers 122 kDa-HIII-fwd (CCCG-GTTGTGGAACGAAGG) and 122 kDa-BsrGI-rev (TTTTGTACAATTGGACCCCGCTTCAAC). The resulting fragment containing Pol II^{Act2} and the 122 kDa coding sequence was digested with *Hind*III/*Bsr*GI and inserted into crTMV-CP-GFP using the same sites.

The *KpnI/ScaI* fragment from pICH4351 (containing the promoter and the MT domain) was inserted into the crTMV *122 kDa* construct digested with *KpnI/SmaI* resulting in a plasmid containing the 122 kDa methyl-transferase (MT) domain and named Pol II^{Act2}-*122 kDa MT*.

All stages of cloning were performed using Fermentas (Lithuania) enzymes.

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Agroinjection. A 500 µl sample of an overnight *A. tumefaciens* (GV-3101 strain) culture was sedimented at 2300g for 5 min, and the pellet was resuspended in a solution containing 10 mM MES (pH 5.5) and 10 mM MgCl₂ to a final OD₆₀₀ = 0.1. When a combination of different strains was used, aliquots of each strain with OD₆₀₀ = 0.2–0.3 were mixed. Leaves of greenhouse grown *Nicotiana benthamiana* plants were injected with this suspension using a syringe without a needle [16, 27].

SDS-PAGE and Western blot analysis. Total soluble protein was extracted from agroinfiltrated *N. benthamiana* leaves. Samples were ground with Celite in phosphate buffered saline (PBS). Crude leaf extracts were resolved on 12% SDS-polyacrylamide gel using Laemmli's buffer system. For Western blot analysis, fractionated proteins were transferred to Hybond-P PVDF membranes (GE Healthcare, UK), blocked with 5% skim milk (Fluka, Germany) in TBS (Tris-buffered saline supplemented with 0.1% Tween-20), and probed with GFP-specific HRP-conjugated antibodies (Rockland Immunochemicals, USA) diluted 1 : 15,000 in TBS. The Western blot was developed with ECL detection reagent (GE Healthcare). Densitometric analysis was performed with Scion Image software (Scion Corp.).

GFP imaging. GFP fluorescence in inoculated leaves was monitored by illumination with a handheld UV source (DESAGA). Fluorescence microscopy was performed using a Zeiss Axiovert 200M microscope (Zeiss, Germany) with two sets of filters, BP470/40 (excitation) and BP525/50 (emission).

GFP fluorescence measurement. Leaf tissue (50 mg) was homogenized in 200 µl of GFP-extraction buffer (200 mM NaCl, 10 mM Tris-HCl, pH 8.0). After centrifugation, the volume of supernatant was adjusted to 1 ml. GFP fluorescence was measured using a QUANTTECH fluorimeter (Barnstead International, USA) equipped with filters NB390 (excitation) and NB520 (emission).

RNA extraction. Total RNA was isolated from plant tissue using TriReagent (MRC, USA) according to the manufacturer's protocol.

Reverse transcription and real-time qPCR analysis. Total cDNA was obtained by annealing 2 µg of denatured total RNA with 0.1 µg of random hexamers and 2 pmol of GFP-RT primer followed by incubation with 200 units of Superscript II reverse-transcriptase (Invitrogen, USA) according to the manufacturer's protocol. Real-time qPCR was carried out using the iCycler iQ real-time qPCR detection system (Bio-Rad, USA). For the detection of target genes, the Eva Green master mix (Syntol, Russia) was used according to the manufacturer's instructions. Each sample was run in triplicate, and a non-template control was added to each run. Target gene mRNA levels were corrected by comparison with RNA levels for the reference gene (18S rRNA). The following oligonucleotides were used: GFP-F1_RT (GCAGAAGAACG-

GCATCAAG), GFP-R1_RT (GCTCAGGTAGTG-GTTGTCG), GFP-RT (CTCGTCCATGCCGTGAGT-GAT), 18S_F1 (ACGGCTACCACATCCAAG), and 18S_R1 (ACTCATTCCAATTACCAGACTC).

RESULTS AND DISCUSSION

Construction of Pol I^{rRNA}-crTMV-GFP vector. The crTMV strain was used to construct the infectious cDNA vectors (Fig. 1a) under control of the *B. oleracea* rRNA promoter (Pol I^{rRNA}) and the *Saccharomyces cerevisiae* terminator. The crTMV genomic RNA is 6312 nt in length, has a cap on its 5'-end, and contains four open reading frames (ORFs). Two components of the replicase complex, 122 and 178 kDa, are encoded by the 5'-proximal ORFs and translated from the viral genomic RNA. The movement protein (MP) and the coat protein (CP) genes are expressed from subgenomic RNAs (sgRNA) that are transcribed from subgenomic promoters in the negative strand of genomic RNA.

To determine if a Pol I promoter can direct the synthesis of crTMV genomic RNA, we constructed a plasmid containing the full-length cDNA of crTMV with GFP instead of the CP gene, designated Pol I^{rRNA}-crTMV-GFP (Fig. 1a, upper). To indicate the cells in which viral replication was initiated, the vector was constructed so that GFP was expressed from the sgRNA made by the viral replicase in infected cells. We did not observe visible fluorescence sites (see, for example, Fig. 3c, site 1), but microscopic study revealed a few clusters of fluorescent cells (Fig. 1b). However, the level of GFP expression was low compared to an analogous vector, Pol II^{Act2}-crTMV-GFP [28], in which GFP expression was under the control of the Pol II actin promoter (*Arabidopsis thaliana* Act2 promoter).

Thus, the Pol I^{rRNA}-crTMV-GFP vector directed GFP synthesis in agroinjected leaves but with low efficiency. This conclusion was confirmed by Western blot analysis of total soluble protein from the agroinjected leaves developed with GFP-specific antibodies (Fig. 1c, lanes 1 and 2).

To verify that transcription of Pol I^{rRNA}-crTMV-GFP was performed by Pol I and not Pol II, we constructed a vector (Pol I^{rRNA}-crTMV(intr)-GFP) containing two *A. thaliana* introns in the 5'-proximal part of the ORF encoding a 122 kDa protein (Fig. 1a, lower). Normally, when transcription is performed by Pol II, these introns are excised during splicing, producing the mRNA from which the fully functional proteins of the replicase complex (122 and 178 kDa) are synthesized. However, if the introns are not removed correctly, the 122 kDa ORF is interrupted by stop codons, no functional 122 and 178 kDa proteins can be synthesized, and no viral vector replication can occur. In contrast, the Pol I export route does not include splicing, so intron insertion into crTMV

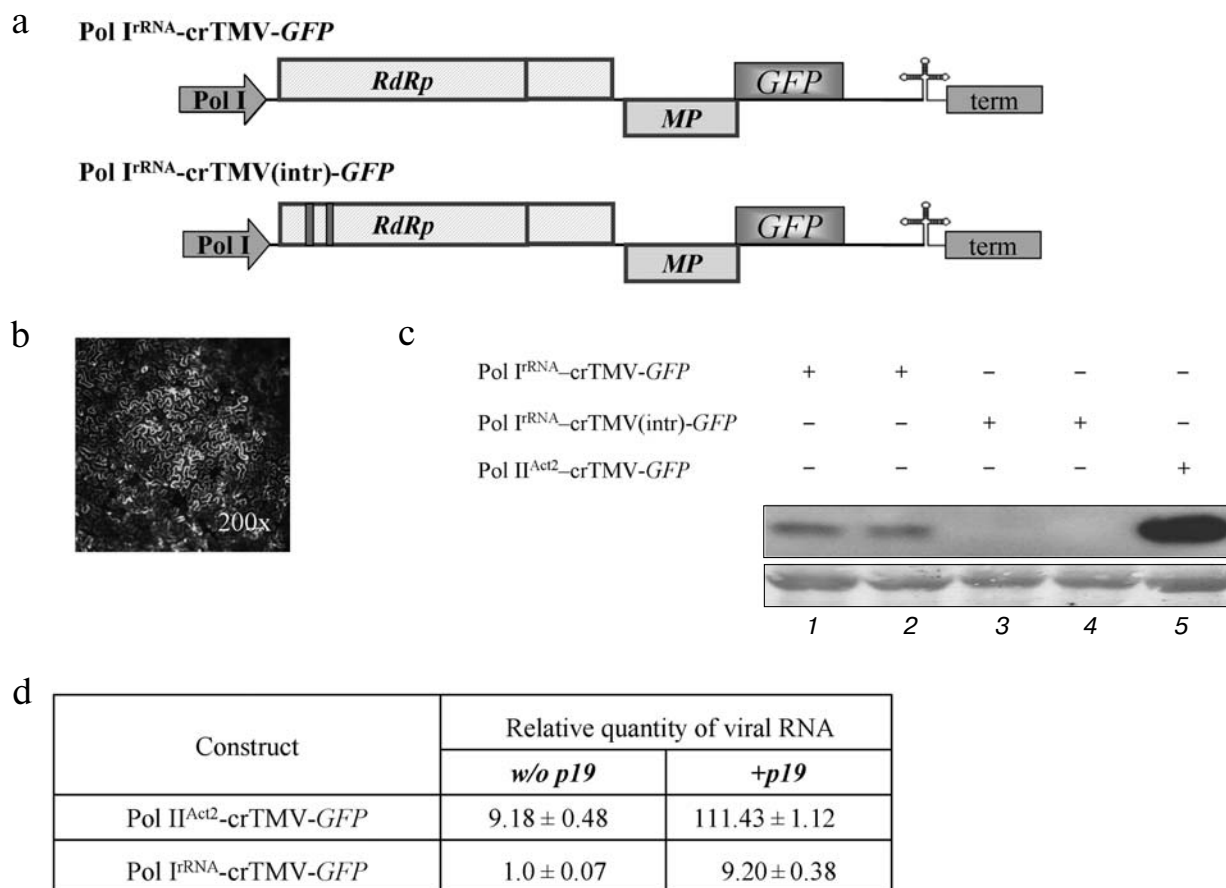


Fig. 1. The Pol I^{rRNA}-promoter-based crTMV vector directs GFP synthesis in *N. benthamiana* leaves. **a)** Schematic of the Pol I^{rRNA}-crTMV-GFP (upper) and intron-containing Pol I^{rRNA}-crTMV(intr)-GFP (lower) vectors. Pol I, *B. oleracea* rRNA promoter; *RdRp*, RNA-dependent RNA polymerase gene; *MP*, movement protein gene; *GFP*, green fluorescent protein gene; term, yeast Pol I transcription terminator. **b)** Fluorescence microscopy photograph of a group of cells under UV light 5 days after inoculation with Pol I^{rRNA}-crTMV-GFP. **c)** Western blot analysis of GFP in the total soluble protein from *N. benthamiana* leaves after agroinjection with Pol I^{rRNA}-crTMV-GFP (lanes 1 and 2), Pol I^{rRNA}-crTMV(intr)-GFP (lanes 3 and 4), and Pol II^{Act2}-crTMV-GFP (lane 5). The lower panel is the protein loading control stained with amido-black. **d)** Relative quantity of GFP mRNA, determined by real-time qPCR in leaf areas after agroinjection with Pol I^{rRNA}- or Pol II^{Act2}-directed TMV-GFP with (+) or without (w/o) the TBSV *p19* silencing suppressor.

genomic RNA abolishes either its RNA export or synthesis of the replicase complex. No fluorescence was detected in leaf areas agroinjected with Pol I^{rRNA}-crTMV(intr)-GFP, and Western blot analysis revealed no GFP production from that viral vector (Fig. 1c, lanes 3 and 4).

The assessment of viral RNA accumulation by real-time PCR showed that the amount of GFP mRNA from Pol I^{rRNA}-crTMV-GFP is approximately 9-fold lower than from Pol II^{Act2}-crTMV-GFP (Fig. 1d, column 1). Our results also showed that co-delivery of the plasmid 35S-*p19* [27] encoding the silencing suppressor, p19, of tomato bushy stunt virus (TBSV) resulted in an approximately 9-fold increase in viral vector RNA accumulation (Fig. 1d, column 2). To explain this result, we suggested that because RNA synthesized by Pol I does not carry any "shield" (a cap, for example) on the 5'-end, these RNAs could be the target of different cellular exonucleases. Moreover, any viral RNA entering the plant cell activates

the host defense system and switches on virus-induced gene silencing (VIGS) that leads to degradation of foreign RNA. Viral RNA protection and suppression of VIGS, especially during the first round of replicase synthesis, may facilitate the subsequent start of replication and thus the increase in GFP production.

We conclude that Pol I-directed transcription may result in the appearance of crTMV RNA in the cytoplasm and that TBSV *p19* stabilizes Pol I-directed viral progeny RNA.

IRES_{CP,148} enhances Pol I-directed crTMV reproduction. The main drawbacks of a Pol I promoter-based system for positive RNA viruses are the inability to make capped, sense viral transcripts to protect the RNA from cellular exonucleases and the inability to recruit translation initiation factors. We suggested that insertion of an internal ribosome entry site (IRES) could partially compensate for the lack of a cap. To test that hypothesis, we

exploited the crTMV IRES_{CP148} [29, 30]. In the case of the crTMV-based viral vector, it was not clear whether the IRES should be inserted at the 5'-end of the genomic RNA or between the 5'-untranslated region (omega, Ω) and the replicase coding sequence. Two IRES-containing constructs were created (Fig. 2a). In the first construct, Pol I^{rRNA}-IRES3'-crTMV-GFP, the IRES_{CP148} was inserted between omega and the replicase gene because the 5'-

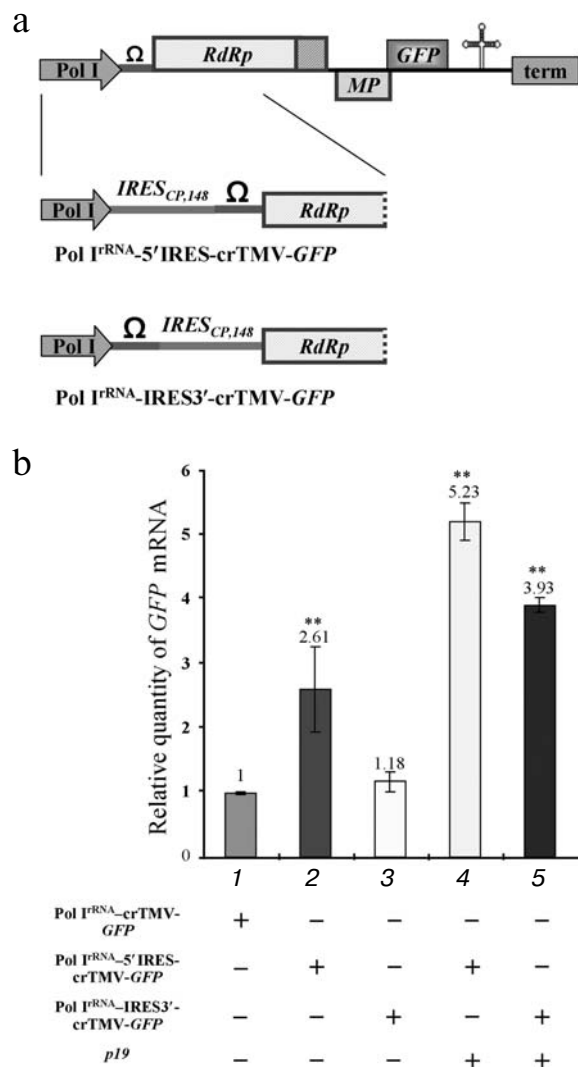


Fig. 2. The crTMV IRES_{CP148} is able to partially compensate for the lack of the viral RNA cap. **a)** Schematic representation of the Pol I^{rRNA}-promoter-based crTMV vectors with the IRES_{CP148} in two positions, upstream (Pol I^{rRNA}-5'IRES-crTMV-GFP) and downstream (Pol I^{rRNA}-IRES3'-crTMV-GFP) of the omega (Ω) sequence. Pol I, rRNA promoter; RdRp, RNA-dependent RNA polymerase gene; MP, movement protein gene; GFP, green fluorescent protein gene; term, yeast Pol I transcription terminator. **b)** Relative quantity of GFP mRNA, determined by real-time qPCR in leaf areas after agroinjection with the IRES_{CP148}-containing Pol I^{rRNA}-based vectors. ** $P < 0.05$ (unpaired two-tailed Student's t -test).

untranslated region should not be important for ribosome internal entry. The infectivity of that viral vector seems to be slightly higher than that of the basic construct, Pol I^{rRNA}-crTMV-GFP, measured by real-time qPCR (Fig. 2b, graph bar 3). The negligible increase in infectivity may be due to the IRES in this position impairing replicase binding to the *cis*-element(s) required for replication. Inserting the IRES_{CP148} just upstream of omega, so as not to interrupt the natural coding sequence of the viral genomic RNA, increased infectivity of the resulting construct, Pol I^{rRNA}-5'IRES-crTMV-GFP, 2.5-fold (Fig. 2b, graph bar 2). Silencing suppression after *p19* co-injection increased the accumulation of RNA directed by both of the IRES-containing constructs more than 2-fold (Fig. 2b, graph bars 4 and 5). Thus, the IRES_{CP148} is able to partially compensate for the lack of the viral RNA cap.

The MT domain of the crTMV 122 kDa gene enhances Pol I-directed crTMV reproduction. It is well known that the methyltransferase activity of the replicase protein 126 kDa TMV U1 is responsible for capping at the first step of viral genomic RNA replication [14, 15]. We assessed whether the crTMV capping enzyme could stimulate viral reproduction in *trans* by protecting viral genomic RNA from exonucleases by adding a cap-structure at its 5'-end. To do so, we constructed a plasmid containing the crTMV 122 kDa gene under control of the Pol II^{Act2}-promoter (Fig. 3a). Joint agroinjection of the Pol I^{rRNA}-crTMV-GFP vector and Pol II^{Act2}-122 kDa may result in only a slight (15–20%) increase in GFP production, measured by densitometry of the band on Western blots (Fig. 3b, graph bar 3). Western blot analysis and densitometry demonstrated more than a 100-fold stimulation of GFP expression from Pol I^{rRNA}-crTMV-GFP when the 122 kDa gene was accompanied by the *p19* gene (Fig. 3b, graph bar 4). In contrast, *p19* alone increased GFP expression only approximately 8-fold (Fig. 3b, graph bar 2). These results were also confirmed by the visual detection of GFP expression in leaf sections (Fig. 3c). Thus, *p19*-mediated protection from the plant defense systems of both Pol I^{rRNA}-directed crTMV-GFP and Pol II-directed 122 kDa RNAs strongly increased the effect achieved by 122 kDa co-expression. Taking into account that Pol I^{rRNA}-crTMV-GFP-mediated GFP production level in the presence of *p19* is 9-fold lower than Pol II^{Act2}-crTMV-GFP-mediated (Fig. 1d), we claim that in combination with the capping enzyme and *p19* (Fig. 3b, No. 4) Pol I^{rRNA}-crTMV-GFP provides the comparable with Pol II^{Act2}-crTMV-GFP level of GFP expression.

To show that the 122 kDa enhancer effect on Pol I^{rRNA}-crTMV-GFP is determined by its N-terminal methyltransferase (MT) domain, which is responsible for capping, we created an *A. thaliana* Act2 promoter-based construct, Pol II^{Act2}-122 kDa MT, encoding the MT domain of the crTMV replicase (Fig. 4a). The truncated 122 kDa gene with the MT domain retained its enhancer properties (Fig. 4b) as confirmed by fluorescence quantification of GFP

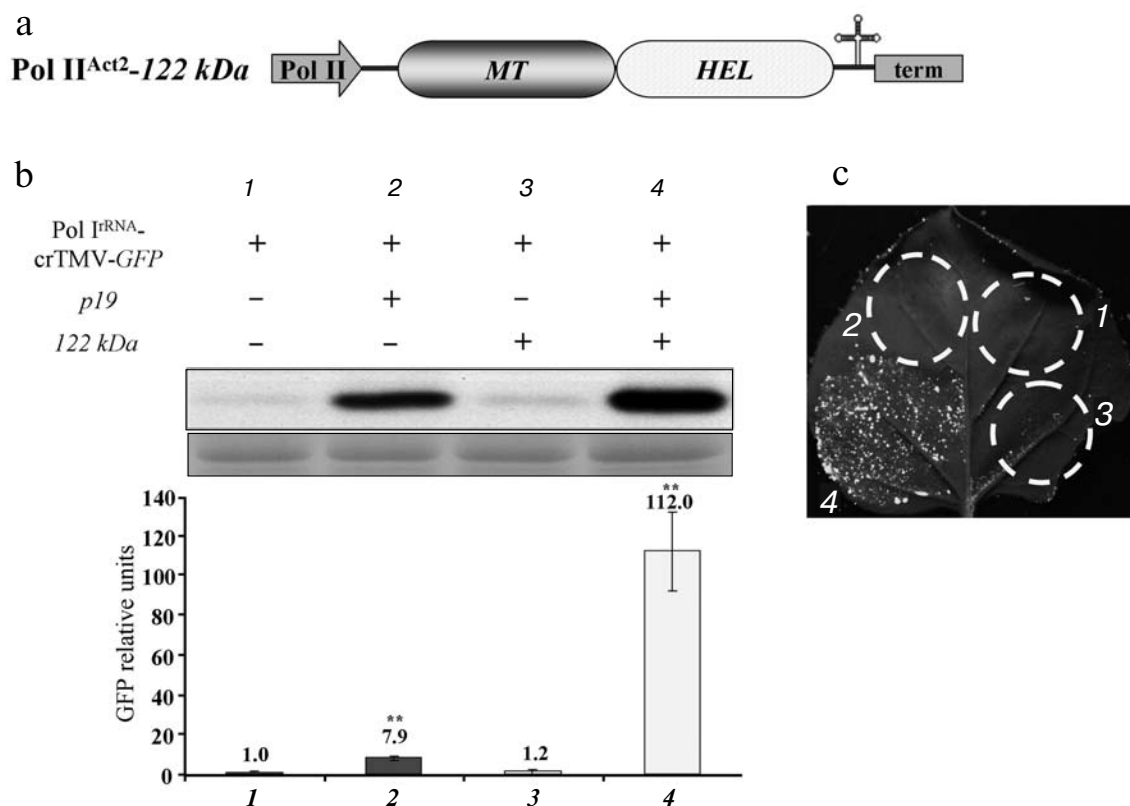


Fig. 3. The 122 kDa capping enzyme and TBSV p19 silencing suppressor stimulate Pol I^{rRNA}-directed crTMV-GFP reproduction. **a)** Schematic of the vector Pr^{Act2}-122 kDa. Pol II, *A. thaliana* Act2 promoter; MT, methyltransferase domain; HEL, helicase domain; term, *nos*-terminator of transcription. **b)** Western blot (upper) of GFP in the total soluble protein from *N. benthamiana* leaves 5 days after agroinjection and the relative density of GFP bands of five independent experiments (bottom). The lower panel is the protein loading control stained with amido-black. ** $P < 0.05$ (Student's *t*-test). **c)** Visualization of GFP expression under UV light 5 days after co-agroinjection with GFP-encoding crTMV constructs.

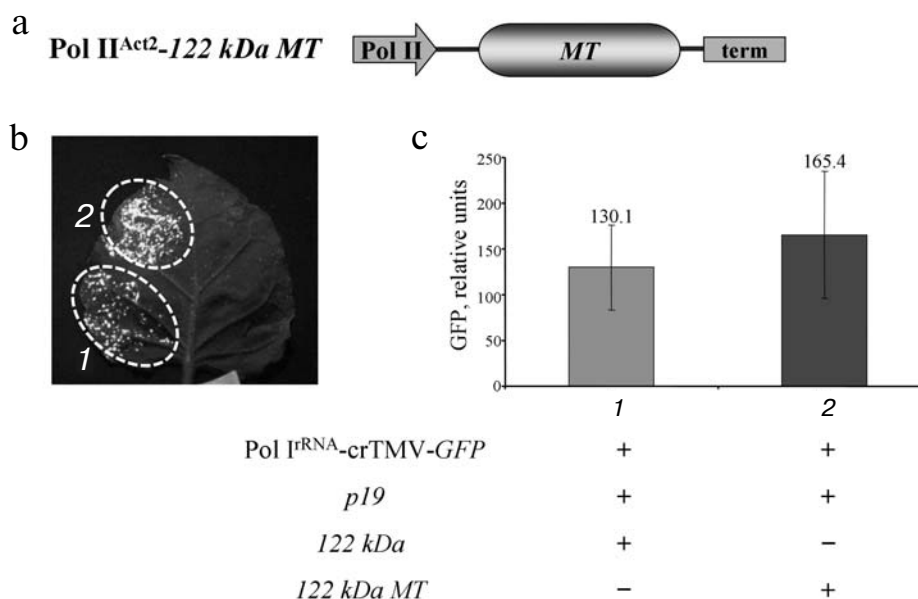


Fig. 4. The crTMV 122 kDa methyltransferase domain is responsible for enhancement of Pol I^{rRNA}-directed crTMV-GFP reproduction. **a)** Schematic of the vector, Pol II^{Act2}-122 kDa MT. Act2, *A. thaliana* Act2 promoter; MT, methyltransferase domain sequence of 122 kDa encoding gene; term, *nos*-terminator. **b)** GFP visualization in *N. benthamiana* leaves 5 days after agroinjection. **c)** GFP fluorimetric analysis. GFP relative units are the result of five independent experiments. $P > 0.05$ (Student's *t*-test).

expression (Fig. 4c). These data demonstrate that the *MT domain* of the crTMV 122 kDa protein is responsible for enhancing Pol I-directed crTMV reproduction.

Collectively, our results show that a simple GFP-encoding crTMV vector based on an rRNA transcriptional cassette (promoter and terminator) is able to produce genomic viral RNA sufficient for crTMV replication despite the lack of RNA capping. Pol I transcripts resulted in GFP production that became significant after viral RNA capping and stabilization. Our transient system provided GFP yields comparable to those of Pol II promoter-based vectors.

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