Plant Mol Biol (2010) 74:591–603
DOI 10.1007/s11103-010-9700-x

Pol II-directed short RNAs suppress the nuclear export of mRNA

Tatiana V. Komarova · Anton M. Schwartz · Olga Y. Frolova · Anna S. Zvereva · Yuri Y. Gleba · Vitaly Citovsky · Yuri L. Dorokhov

Received: 26 March 2010 / Accepted: 28 September 2010 / Published online: 17 October 2010

Abstract The synthesis and subsequent nuclear export of non-coding RNA (ncRNA) directed by RNA polymerase (Pol) II is very sensitive to abiotic and biotic external stimuli including pathogen challenges. To assess whether stress-induced ncRNAs may suppress the nuclear export of mRNA, we exploited the ability of *Agrobacterium tumefaciens* to co-deliver Pol I, II and III promoter-based vectors for the transcription of short (s) ncRNAs, *GFP* mRNA or genomic RNA of plant viruses (*Tobacco mosaic virus,* TMV; or *Potato virus X,* PVX) into the nucleus of *Nicotiana benthamiana* cells. We showed that, in contrast to Pol I- and Pol III-derived sncRNAs, all tested Pol II-derived sncRNAs (U6 RNA, tRNA or artificial RNAs) resulted in decreased expression of *GFP* and host mRNA. The level of this inhibitory effect depended on the non-coding transcript length and promoter strength. Short coding RNA (scRNA) can also compete with mRNA for nuclear export. We showed that scRNA, an artificial 117-nt short sequence encoding **E**lastin-**L**ike peptide element tandems with **F**LAG sequence (ELF) and the 318-nt *N. benthamiana* antimicrobial peptide thionin (defensin) gene efficiently decreased GFP expression. The stress-induced export of Pol II-derived sncRNA and scRNA into the cytoplasm via the mRNA export pathway may block nucleocytoplasmic traffic including the export of mRNA responsible for antivirus protection. Consistent with this model, we observed that Pol II-derived sncRNAs as well as scRNA, thionin and ELF strongly enhanced the cytoplasmic reproduction of TMV and PVX RNA.

Keywords Non-coding RNAs · Nuclear export · mRNA · Plant virus · RNA polymerase II · Short coding RNA

Introduction

The evolutionarily conserved global alteration of the nuclear mRNA export pathway in response to environmental stress

Tatiana V. Komarova and Anton M. Schwartz contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s11103-010-9700-x) contains supplementary material, which is available to authorized users.

T. V. Komarova · O. Y. Frolova · A. S. Zvereva · Y. L. Dorokhov (✉)
A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Leninsky Gory 1, Laboratory Building A, 119992 Moscow, Russia
e-mail: dorokhov@genebee.msu.su

A. M. Schwartz · O. Y. Frolova · Y. L. Dorokhov
N. I. Vavilov Institute of General Genetics, Russian Academy of Science, 119992 Moscow, Russia

Y. Y. Gleba
Nomad Bioscience GmbH, Biozentrum Halle, Weinbergweg 22, 06120 Halle, Saale, Germany

V. Citovsky
Department of Biochemistry and Cell Biology, State University of New York, Stony Brook, NY 11794, USA

Present Address:
A. S. Zvereva
Botanical Institute, University of Basel, Schönbeinstr. 6, 4056 Basel, Switzerland

A. S. Zvereva
e-mail: zvereva@gmail.com

is well established (Bond 2006; Gallouzi et al. 2000; Izawa et al. 2005; Krebber et al. 1999; Stewart 2010). For example, transcripts from genes encoding heat shock proteins (hsp) are exported in response to heat shock, whereas most non-hsp poly(A+) mRNAs accumulate in the nucleus (Saavedra et al. 1996,1997). A mechanism that coordinates the transcription and export of hsp mRNAs necessary for the cellular stress responses has been suggested (Carmody et al. 2010).

Like mRNA, non-coding RNA (ncRNA) exported from the nucleus play a role in temperature stress accommodation (Chinnusamy et al. 2008, 2010). ncRNAs are transcribed by one of three eukaryotic nuclear RNA polymerases (Pols I, II, III). The set of transcripts synthesised by Pol II includes protein-coding mRNAs and several ncRNAs such as small nuclear, nucleolar and micro (mi) RNAs. The genomic organisation of ncRNAs varies greatly, and they can be expressed both from intergenic regions and protein-coding sequences (Brown et al. 2008). Plant ncRNA biogenesis involves the synthesis of primary transcripts, processing by Dicer-like enzyme 1 (DCL1) in the nucleus (Vaucheret 2006; Xie et al. 2004) and export into the cytoplasm (Yelina et al. 2010), which involves HASTY (a plant ortholog of exportin 5) (Park et al. 2005) and Cajal bodies (Li et al. 2006). Recent evidence suggests that the synthesis and subsequent nuclear export of ncRNAs is sensitive to external stimuli (Ben Amor et al. 2009; Liu et al. 2008; Voinnet 2008) including pathogen challenge (Bazzini et al. 2007; Navarro et al. 2008).

To elucidate the role of ncRNA export in mRNA expression we used *Agrobacterium*-mediated delivery of Pol I, II and III promoter-based vectors encoding short (s) ncRNAs, *GFP* mRNA, or genomic RNAs of plant viruses, such as *Tobacco mosaic virus* (TMV) and *Potato virus X* (PVX), into the plant cell nucleus. We showed that Pol II-derived sncRNAs decreased the expression of GFP and host proteins and increased the reproduction of TMV and PVX RNA. Moreover, short coding RNAs (scRNAs), the thionin gene and an artificial short sequence encoding **E**lastin-**L**ike peptide element tandems with **F**LAG sequence (ELF) efficiently decreased the expression of GFP and stimulated cytoplasmic virus reproduction.

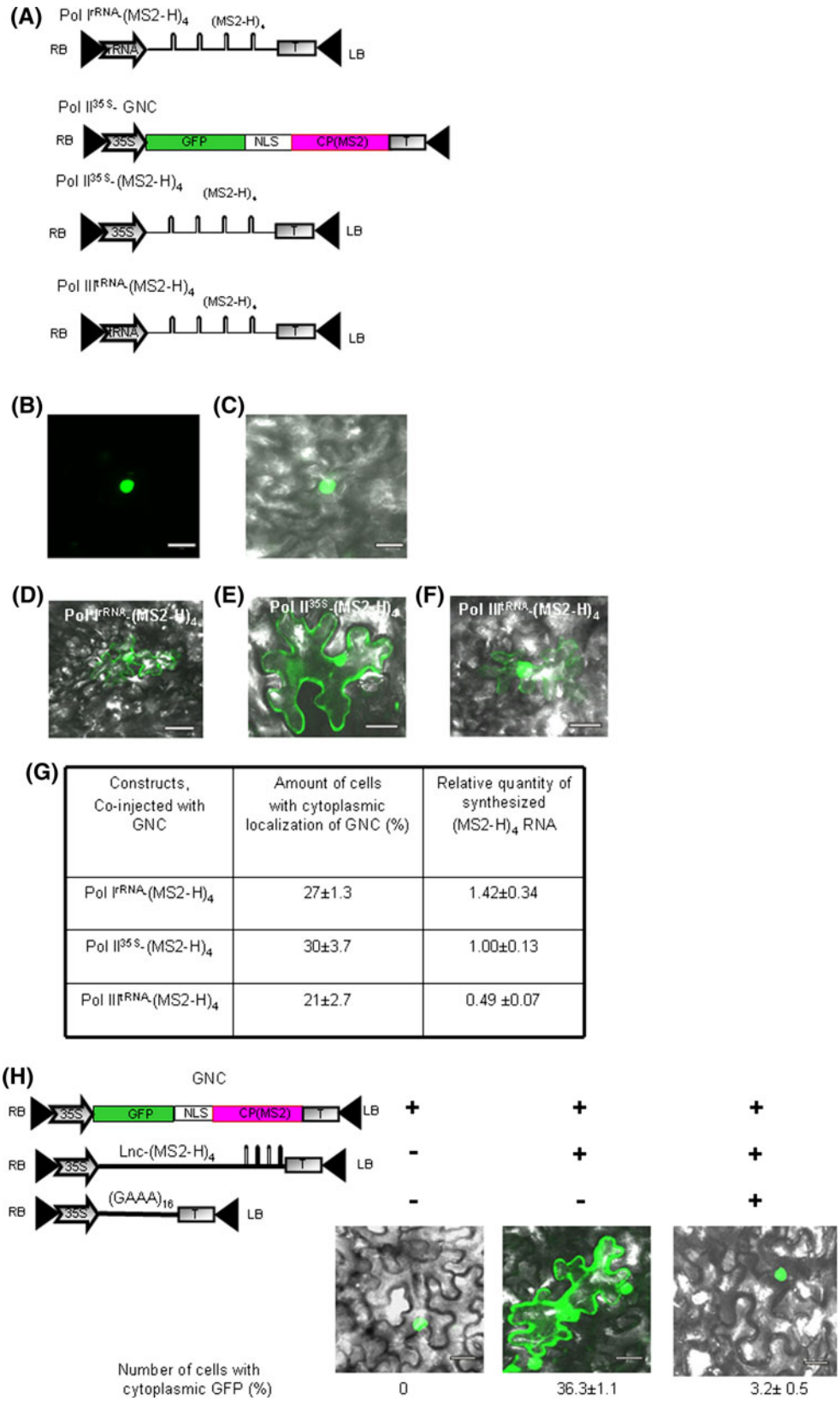
Materials and methods

Plasmid constructs

The plasmids pBor2, which contains the *Brassica oleracea* rRNA gene promoter sequence from −518 to +106, and pNtY1, which contains the *Nicotiana rustica* cytoplasmic tRNA-tyr gene, were used in the Pol I and Pol III promoter-based vectors, respectively. For plasmid (pMS2H), which encodes the MS2 hairpin unit (5′ACA-AGAGGATCACCCATGT3′), PCR with overlapping primers MS2-1 and MS2-2 was performed. A previously described plasmid encoding the $(GAAA)_{16}$ unit (Dorokhov et al. 2002) was used to produce Bin19-based vectors $(GAAA)_{n,}$ where n ranged from 8 to 128. For the promoter-based vector encoding *A. thaliana* RNA U6, PCR was performed with the overlapping primers U6-1p, U6-1 m, U6-2p and U6-2 m. The viral vectors crTMV:GFP (pICH4351), TMV:GFP(intr) (pICH18711), 35S-*GFP* and 35S-*GFPi* have been previously described (Marillonnet et al. 2005). For PVX-W-$(GAAA)_{16}$ and PVX-Mut-$(GAAA)_{16}$, previously described plasmids (Komarova et al. 2006) were utilised. Overlapping PCR with the miR159-specific primer 159/171 and plasmid pBICmiR171prec, which was kindly provided by Dr. T. Okuno (Kyoto University), was used for the 35S promoter-based vector encoding the precursor of chimeric miRNA171/159. The binary constructs pGNC, which encodes GFP fused to NLS and MS2 CP, pBor2 and pNtY1 were kindly provided by Drs. A. Simon (University of Maryland, College Park), C. S. Pikaard (Washington Unversity St. Louis), and H. Beier (University of Würzburg), respectively.

An ELF sequence was created with overlap PCR, and the product was cloned into a 35S-based vector by replacing the GFP-RFP cassette with the ELF sequence in the 35S-GFP-RFP vector (Schwartz et al. 2006) using NcoI-XhoI sites, resulting in the 35S-ELF plasmid. An intron-containing 35S-thionin(i) construct was created based on the natural *N. benthamiana* thionin sequence (EMBL accession number FR686584), and 5′-NcoI and 3′-XhoI sites were introduced via PCR. Its intronless analogue, 35S-thionin, was created with overlap PCR, and both products were cloned into the 35S-based vector by replacing the GFP-RFP cassette with the thionin(i) or thionin sequence in a 35S-GFP-RFP vector (Schwartz et al. 2006) using NcoI-XhoI sites.

The following PCR primers were used in this study: *MS2-1,* 5′CTGAGGATCCGAATTCTGTAACTTAAGGACCATCAGGCCTTAAG3′; *MS2-2*, 5′CTGAAGATCTCAATTGCTTAAGGCCTGATGGTCCTTAAG3′; *159/171*, 5′TTTGGATTGAAGGGAGCTCTA3′; *U6-1p*, 5′GATCGGTACCGTCTCTTCGGAGACATCCGATAAAATTGGA3′; *U6-1 m*, 5′GGGGCCATGCTAATCTTCTCTGTATCGTTCCAATTTTATCGG3′; *U6-2p,* 5′ATTAGCATGGCCCCTGCGCAAGGATGACACGCACAAATCG3′; *U6-2 m*, 5′GATCGTCGACAAAATTTGGACCATTTCTCGATTTGTGCGTGT3′; *ELF-D1*, 5′CCATGGCAGTTGGTGTTCCCGGTGTCGCTGTTCCAGGACTTGGAG3′; *ELF-D3*, 5′GTGCCAGGGGTAGCCGTCCCTGGCTTGGGAGTT



Constructs, Co-injected with GNC	Amount of cells with cytoplasmic localization of GNC (%)	Relative quantity of synthesized (MS2-H)$_4$ RNA
Pol I^{rRNA}-(MS2-H)$_4$	27±1.3	1.42±0.34
Pol II^{35S}-(MS2-H)$_4$	30±3.7	1.00±0.13
Pol III^{tRNA}-(MS2-H)$_4$	21±2.7	0.49 ±0.07

Fig. 1 Detection of Pol I^{rRNA}-, Pol II^{35S}-, or Pol III^{tRNA}-derived sncRNAs in the plant cell cytoplasm. (**a**) Schematic structure of vectors. Pol II^{35S}-GNC, 35S-based vector encoding GFP fused with the SV40 nuclear localisation signal (NLS) and MS2 coat protein (CP); Pol I^{rRNA}-(MS2-H)$_4$, Pol II^{35S}-(MS2-H)$_4$ and Pol III^{tRNA}-(MS2-H)$_4$ binary vectors encoding an MS2 hairpin tetramer (MS2-H)$_4$. LB and RB are the left and right T-DNA borders, respectively. (**b–f**) Visualisation of GFP expression in epidermal cells of leafs co-agroinjected with GFP:NLS:CP$_{MS2}$ (**b**, **c**), Pol II^{35S}-GNC and Pol I^{rRNA}-(MS2-H)$_4$ (**d**), Pol II^{35S}-(MS2-H)$_4$ (**e**) and Pol III^{tRNA}-(MS2-H)$_4$ (**f**) with their confocal images (**b**) and their overlays with the respective false-transmission images. (**c–f**) Projections of several confocal sections superimposed on a bright field image of the same cell. Bars = 20 μm. (**g**) Numbers of cells with cytoplasmic localisation of GFP:NLS:CP$_{MS2}$ and the relative quantity of (MS2-H)$_4$ RNA determined by real-time PCR. (**h**) GFP relocalisation from the nucleus to the cytoplasm in leaves co-agroinjected with Pol II^{35S}-GNC, Pol II^{35S}-Lnc-(MS2-H)$_4$ and Pol II^{35S}-(GAAA)$_{16}$. Numbers of cells with cytoplasmic localisation of GFP:NLS:CP$_{MS2}$ are indicated as a percent of the total number of GFP-expressing cells

CCGGACTATAAG3′; *ELF-R2*, 5′GGCTACCCCTGG CACGCCAGCACCAGGCACTCCAAGTCCTGGAAC3′; *ELF-R4*, 5′CTCGAGTTATTTATCATCATCATCCTTAT AGTCCGGAACTCCC3′; *Thi-dir2,* 5′GTTGCCTATGAT GTGGAAGCTAAAAATTGC3′; and *Thi-rev2*, 5′CTTCC ACATCATAGGCAACAAAGAGCATCA3′.

Agroinjection

Agrobacterium tumefaciens strain GV3101 was transformed with individual binary constructs and grown at 28°C in LB medium supplemented with 50 mg/l rifampicin, 25 mg/l gentamicin and 50 mg/l carbenicillin. *Agrobacterium* cells from a 5-ml overnight culture were collected by centrifugation (10 min, 4,500×*g*), resuspended in 10 mM MES (pH 5.5) buffer supplemented with 10 mM $MgSO_4$, and adjusted to a final OD_{600} of 0.8. Agroinjection was performed on almost fully expanded *N. benthamiana* leaves attached to the intact plant. A bacterial suspension was infiltrated into the leaf tissue using a 5-ml syringe, and the plants were grown in greenhouse conditions at 22°C with 16 h of light.

GFP imaging

The GFP fluorescence in inoculated leaves was monitored by illumination with a handheld UV source (DESAGA). At higher magnifications, GFP fluorescence was detected using a dissecting microscope (Opton IIIRS) equipped with an epifluorescence module. Unless otherwise indicated, the lower epidermal cells of injected leaves were observed 24–26 h after agroinjection. Confocal imaging of GFP-expressing cells was performed using an inverted Carl Zeiss LSM 510 laser-scanning microscope (Jena).

Western blot analyses

Total proteins isolated from leaves were subjected to SDS-polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Amersham). The membranes were probed with affinity-purified rabbit antibodies raised against tobacco BiP. Goat anti-rabbit IgG conjugated to horseradish peroxidase (Sigma) was used as the secondary antibody, and the reaction was visualised by chemiluminescence (ECL system, Amersham Pharmacia).

Quantitative real-time PCR (qRT-PCR) analysis of transcript concentrations

RNA concentrations were measured by spectrophotometry (A260 nm), and the RNA quality was assessed by measuring the A260/A280 nm absorption ratio. After DNase treatment (Fermentas), cDNA was obtained by annealing 2 μg of denatured total RNA with 0.1 μg of random hexamers and 0.1 μg of Oligo-dT following incubation with 200 units of Superscript II Reverse Transcriptase (Invitrogen) for 50 min at 43°C. qRT-PCR was carried out using the iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA, USA). EvaGreen Master Mix (Syntol, Russia) was used according to the manufacturer's instructions to detect target genes. The thermal profile for EvaGreen qRT-PCR included an initial heat denaturing step at 95°C for 3 min and 45 cycles at 95°C for 15 s, an annealing step (see Supplementary Table S1) for 30 s and 72°C for 30 s coupled with fluorescence measurement. Following amplification, the melting curves of PCR products were monitored from 55 to 95°C to determine the specificity of amplification. Each sample was run in triplicate, and no-template controls were included in each run. The PCR efficiency (E) was calculated according to the equation $E = 10(-1/\text{slope})$ by creating the standard curves. Target gene mRNA levels were corrected with the corresponding reference genes (18S and ef-2$\acute{\alpha}$ for *N. tabacum*).

Results

Technique to detect cytoplasmic sncRNAs

To monitor the export of RNA from the nucleus to the cytoplasm, we utilised a method based on the binding of the MS2 bacteriophage coat protein (CP_{MS2}) to a 19-base hairpin (MS2 hairpin) (Zhang and Simon 2003). In this approach, a fusion protein (GNC) containing CP_{MS2}, GFP and a nuclear localisation signal (NLS) is redirected from the nucleus to the cytoplasm in leaves co-agroinjected with RNA containing the MS2 hairpin (Fig. 1a). GFP fused to SV40 NLS and CP_{MS2} accumulates in the cell nucleus (Fig. 1b, c) in *N. benthamiana* leaves agroinjected with the construct that encodes it, Pol II^{35S}-GNC (Fig. 1a).

We then constructed infectious cDNA of TMV U1, Pol II^{Act2}-TMV-$(MS2\text{-}H)_4$ (Fig. S1 A), which directs via the CP subgenomic promoter synthesis of the MS2 hairpin tetramer in the cell cytoplasm. Co-agroinjection of leaves with Pol II^{Act2}-TMV-$(MS2\text{-}H)_4$ and Pol II^{35S}-GNC resulted in the relocation of GFP:NLS:CP_{MS2} to the cytoplasm (Fig S1 B, C), presumably due to the interaction between CP_{MS2} and the cytoplasmic MS2 hairpin. We then constructed Pol I^{rRNA}-, Pol II^{35S}- and Pol III^{tRNA}-based binary vectors encoding the MS2 hairpin tetramer $(MS2\text{-}H)_4$ (Fig. 1a) and assessed the accumulation of the corresponding RNAs using real-time PCR (Fig. 1g, last column). These experiments revealed that co-agroinjection of Pol II^{35S}-GNC with Pol I^{rRNA}-, Pol II^{35S}-, and Pol III^{tRNA}-based $(MS2\text{-}H)_4$ vectors partially redirected GFP:NLS:CP_{MS2} to the cytoplasm (Fig. 1d–f). Thus, this CP_{MS2}-based technique allows the detection of Pol I, Pol II and Pol III promoter-directed sncRNAs in the cytoplasm by redirecting the GFP reporter from the nucleus to the cytoplasm.

Pol II-derived sncRNA and mRNA may share the same nuclear export pathway

Can different types of RNA molecules compete with one another for the nuclear export machinery of the cell? To address this question, we created a series of binary vectors (Fig. 1h) encoding (i) long (> 1,800 nt) non-coding RNA with $(MS2\text{-}H)_4$ at the 3′-terminus [Pol II^{35S}-Lnc-$(MS2\text{-}H)_4$] and (ii) Pol I^{rRNA}-, Pol II^{35S}- and Pol III^{tRNA}-based binary constructs encoding artificial sncRNA sequences composed of 16 GAAA repeats [$(GAAA)_{16}$]. Agroinjection of *N. benthamiana* leaves with Pol II^{35S}-GNC and Pol II^{35S}-Lnc-$(MS2\text{-}H)_4$ resulted in the relocation of GFP:NLS:CP_{MS2} from the nucleus to the cytoplasm (Fig. 1h). When Pol I^{rRNA}-$(GAAA)_{16}$ or Pol III^{tRNA}-$(GAAA)_{16}$ were co-agroinjected with Pol II^{35S}-GNC and Pol II^{35S}-Lnc-$(MS2\text{-}H)_4$, the GFP:NLS:CP_{MS2} reporter remained mostly cytoplasmic (data not shown). However, co-agroinjection of Pol II^{35S}-GNC and Pol II^{35S}-Lnc-$(MS2\text{-}H)_4$ with Pol II^{35S}-$(GAAA)_{16}$ restored the nuclear localisation of GFP:NLS:CP_{MS2}

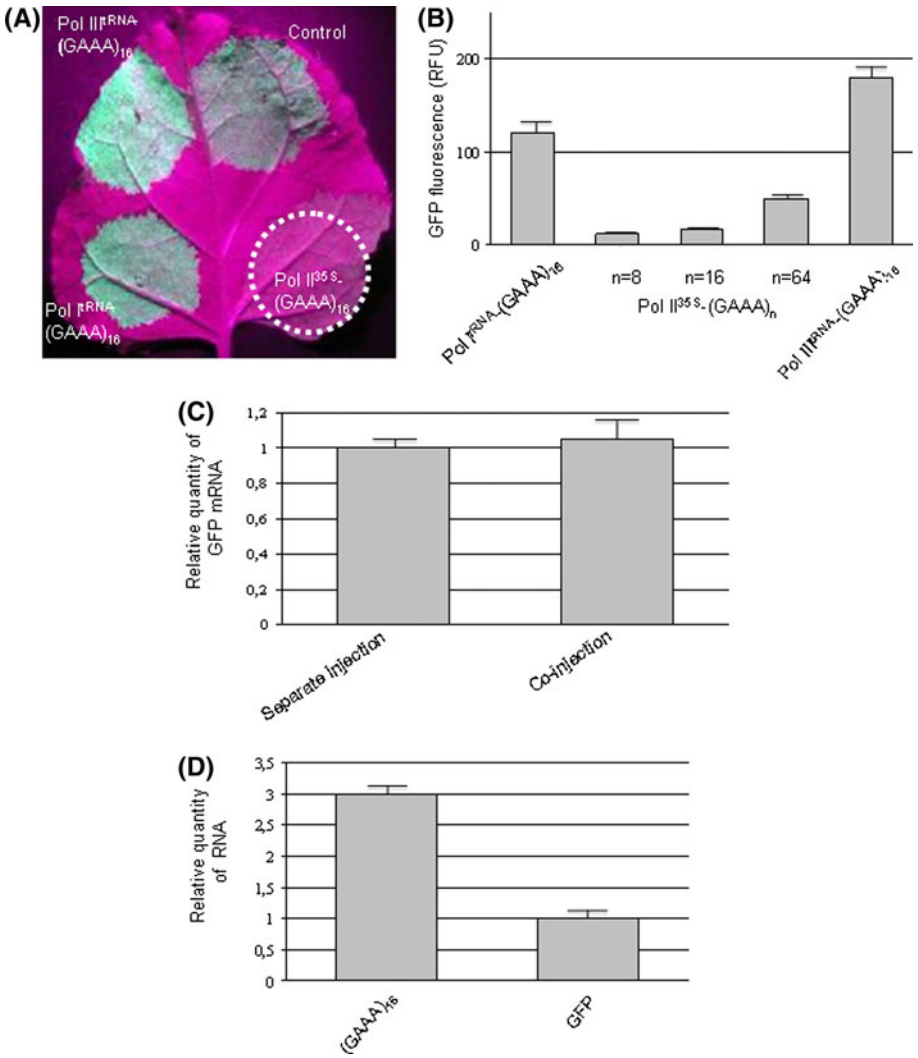


Fig. 2 Pol II promoter-directed sncRNAs suppress GFP accumulation. (**a**) GFP expression 3 days after co-agroinjection of leaf sectors with a GFP-encoding binary construct and constructs encoding $(GAAA)_{16}$ sncRNA under the control of Pol I^{rRNA}-, Pol II^{35S}-, and Pol III^{tRNA}-dependent promoters. Control: GFP-expressing vector co-agroinjected with an empty pBin19 vector. (**b**) Visual detection and fluorimetric analysis of GFP accumulation in experiments described in panel A. The data represent eight independent experiments. Fluorescence measurements are presented in relative light units with the maximal GFP fluorescence, which was observed in co-agroinjection with the empty pBin19, assigned a value of 100. Data were corrected for autofluorescence and represent 5–8 independent experiments with the standard *error bars* indicated. (**c**) Relative quantity of *GFP* mRNA as determined by real-time PCR in leaf areas agroinjected with either the GFP-expressing vector alone (separate injection) or together with Pol II^{35S}-$(GAAA)_{16}$ (co-injection). (**d**) Relative quantity of Pol II^{35S}-derived $(GAAA)_{16}$ sncRNA and *GFP* mRNA as determined by real-time PCR in leaf areas co-agroinjected with the GFP-expressing vector and Pol II^{35S}-$(GAAA)_{16}$

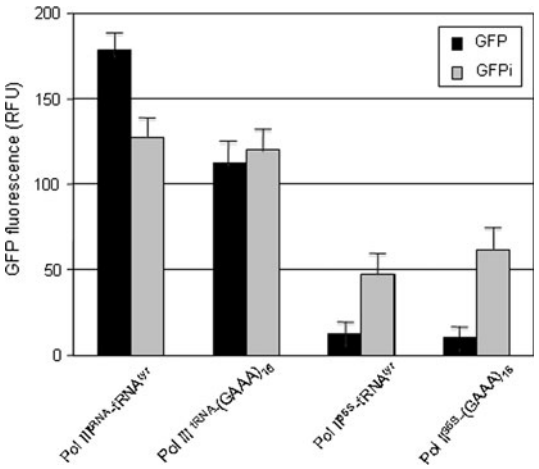


Fig. 3 sncRNA-mediated suppression of GFP expression is blocked by the insertion of an intron in the *GFP* gene. Fluorimetric analysis of GFP accumulation in leaves 3 days after co-agroinjection with Pol II^{35S}-$(GAAA)_{16}$, Pol II^{35S}-$tRNA^{tyr}$, Pol III^{tRNA}-$(GAAA)_{16}$, or Pol III^{tRNA}-$tRNA^{tyr}$ and a binary vector expressing either the *GFP* gene or the intron-containing *GFP* gene (GFPi). Fluorescence measurements are presented in relative light units with the maximal GFP fluorescence, which was observed in co-agroinjection with empty pBin19, assigned a value of 100

(Fig. 1h), suggesting that Pol II^{35S}-$(GAAA)_{16}$ sncRNA may block the export of Pol II^{35S}-Lnc-$(MS2\text{-}H)_4$ RNA from the nucleus to the cytoplasm.

To examine whether the nuclear export of Pol II^{35S}-derived sncRNA may interfere with the nuclear export of mRNA, we used the *GFP* transcript as a reporter for mRNA export. Unlike Pol I^{rRNA}-$(GAAA)_{16}$ or Pol III^{tRNA}-$(GAAA)_{16}$, co-agroinjection of Pol II^{35S}-$(GAAA)_{16}$ dramatically suppressed *GFP* mRNA expression in agro-injected leaves (Fig. 2a). The degree of GFP expression was quantified by fluorescence and revealed twofold to tenfold suppression (Fig. 2b). Two lines of evidence indicate that this effect is not likely the result of simple competition between Pol II^{35S}-$(GAAA)_{16}$-encoded sncRNA and *GFP* mRNA for the cytoplasmic translation machinery of the cell. First, the insertion of an intron into the *GFP* gene (*GFPi*) enhanced its expression after co-injection with Pol II^{35S}-$(GAAA)_{16}$ (Fig. 3) or with Pol II^{35S}-$(MS2\text{-}H)_4$ (Fig. S2). The presence of an intron is not expected to relieve the competition between mRNAs for the translation machinery. Second, GFP expression and the implied *GFP* mRNA nuclear export was not inhibited when the

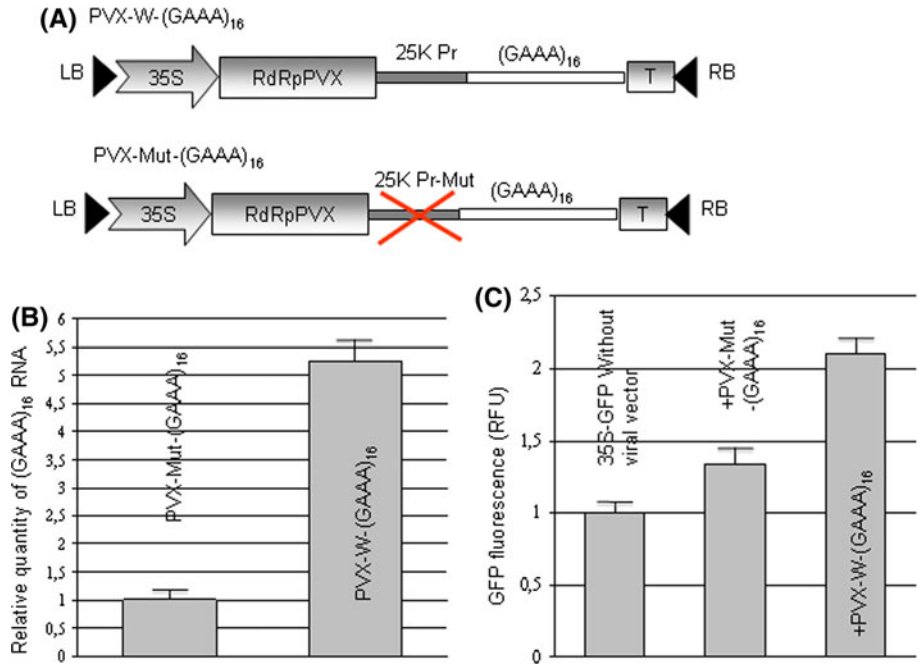


Fig. 4 $(GAAA)_{16}$ sncRNA synthesised in the cell cytoplasm does not affect GFP accumulation. (**a**) Schematic structure of the PVX-W-$(GAAA)_{16}$ and PVX-Mut-$(GAAA)_{16}$ vectors composed of the following elements: LB and RB, the left and right T-DNA borders, respectively; 35S, CaMV promoter; RdRp, RNA-dependent RNA polymerase (viral replicase) gene; 25 K Pr, 25 K PVX subgenomic promoter, 25 K Pr-Mut—inactivated mutated 25 K Pr. (**b**) Relative quantity of $(GAAA)_{16}$ sncRNA as determined by real-time PCR in leaf areas co-agroinjected with PVX-based and GFP binary vectors. (**c**) Fluorimetric analysis of GFP accumulation in leaves 3 days after co-agroinjection with PVX-W-$(GAAA)_{16}$ or PVX-Mut-$(GAAA)_{16}$ and GFP-expressing binary vector. Fluorescence measurements are presented in relative light units with the maximal GFP fluorescence, which was observed in co-agroinjection with empty pBin19, assigned a value of 1.0

$(GAAA)_{16}$ sncRNA was synthesised directly in the cytoplasm from a PVX-based vector (Fig. 4), which is expected to affect nuclear export but not translation directly.

Further support of the idea that Pol II^{35S}-derived sncRNAs suppress mRNA export by competing for the export machinery was acquired by quantifying the amounts of *GFP* mRNA and Pol II^{35S}-derived $(GAAA)_{16}$ sncRNA in agroinjection experiments by real-time RT-PCR. First, GFP RNA was produced in equal amounts in the double and single agroinjections (Fig. 2c), excluding the possibility of competition between 35S promoters for Pol II. Second, preferential accumulation of the Pol II^{35S}-derived $(GAAA)_{16}$ sncRNA compared to the *GFP* mRNA (Fig. 2d) and Lnc-$(MS2\text{-}H)_4$ (Fig. S3) was observed, suggesting that shorter RNAs are produced more efficiently. Thus, the level of GFP expression may inversely depend on the length of the transcript (Fig. 2b). Consistent with this scenario, truncation of the 35S promoter (Fig. 5a) resulted in reduced levels of Pol II^{35S}-derived $(GAAA)_{16}$ sncRNA (Fig. 5b) and higher expression levels of the GFP reporter (Fig. 5c), indicating that the inhibitory effect of sncRNAs on GFP expression depends on the amount of sncRNA.

We produced natural U6 snRNA from Pol I^{rRNA}-, Pol II^{35S}-, or Pol III^{tRNA}-dependent promoters (Fig 6a). Only the Pol II^{35S}-derived U6 snRNA inhibited the accumulation of the GFP reporter (Fig. 6b, c) and the cellular protein BiP (Fig. 6d). Furthermore, the insertion of an intron into the U6 snRNA sequence substantially enhanced this inhibitory effect on BiP (Fig. 6d), confirming that intron-containing pre-mRNAs are more sensitive to the inhibitory effect of intron-containing sncRNAs, most likely because of additional competition for splicing machinery, which is tightly connected to nuclear mRNA export.

Thus, our data suggest that Pol II^{35S}-derived sncRNA and mRNA and U6 snRNA may share the same pathway for nuclear export.

Effect of sncRNAs on the reproduction of plant viral genomic RNA

Because Pol II^{35S}-derived sncRNAs can block the nuclear export of mRNA, they may also interfere with antiviral defence responses, which are known to involve the nuclear export of RNA molecules (Csorba et al. 2007; Lózsa et al. 2008). We tested this hypothesis using binary vectors encoding infectious cDNA clones of crucifer-infecting *Tobacco mosaic virus* (crTMV) (Fig. 7) or *Potato virus X* (PVX) (Fig. S4). Both viral cDNAs contained a *GFP* reporter gene, which facilitated the detection and quantification of viral reproduction (Fig. 7a and Fig. S4). Pol II^{35S}-derived U6 snRNA but not Pol I^{rRNA}- or Pol III^{tRNA}-derived U6 snRNA significantly increased the accumulation of the virus-encoded GFP and, by implication, viral

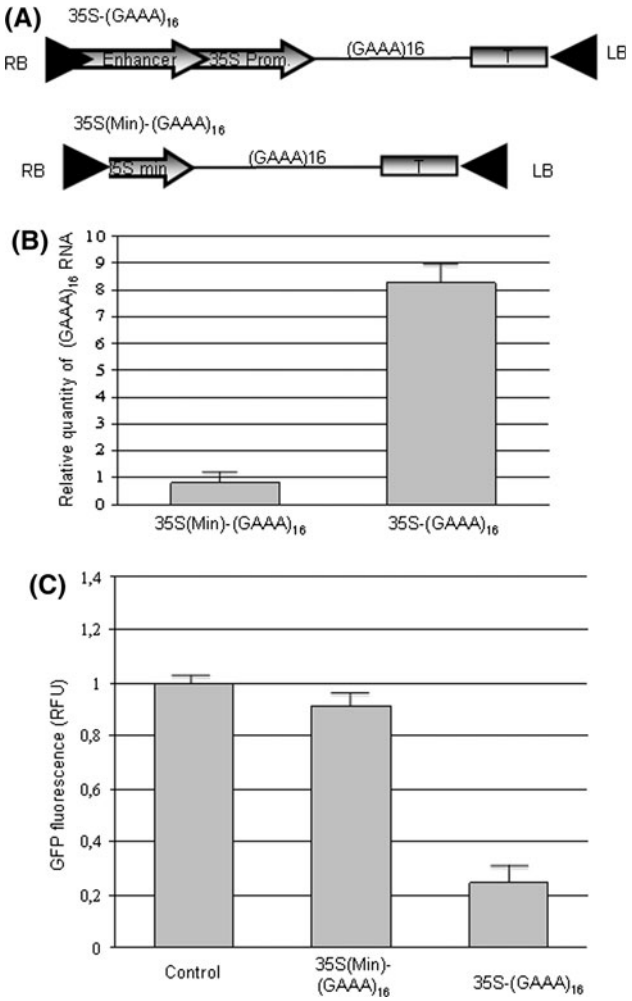


Fig. 5 Inverse relationship between the amount of Pol II^{35S}-derived $(GAAA)_{16}$ sncRNA synthesised under the control of a truncated 35S promoter and GFP accumulation. (**a**) Schematic structure of the 35S-$(GAAA)_{16}$ and 35S(Min)-$(GAAA)_{16}$ binary vector. 35S(Min), truncated 35S promoter. (**b**) Relative quantity of $(GAAA)_{16}$ sncRNA determined by real-time PCR. (**c**) Fluorimetric analysis of GFP accumulation in leaves 3 days after co-agroinjection with 35S-$(GAAA)_{16}$ or 35S(Min)-$(GAAA)_{16}$ and GFP-expressing binary vectors. Fluorescence measurements are presented in relative light units with the maximal GFP fluorescence, which was observed in co-agroinjection with empty pBin19, assigned a value of 1.0 (control)

RNA (Fig. 7a–c). This is consistent with our earlier observations (Dorokhov et al. 2006) that the co-agroinjection of *N. benthamiana* leaves with cDNA encoding the 123-nt precursor of miR171 stimulated the accumulation of *GFP* subgenomic mRNA directed by the TMV-based vector. Here, we tested other sncRNAs including chimeric miRNA171/159, tRNA, and GAAA tandem repeats and showed that the stimulating effect on virus reproduction depended on the length of the sncRNA (Fig. 7d) whereas sncRNA nucleotide sequences or their secondary structures had no influence. Interestingly, the insertion of introns into viral RNA (Fig. 7e), which is known to enhance the

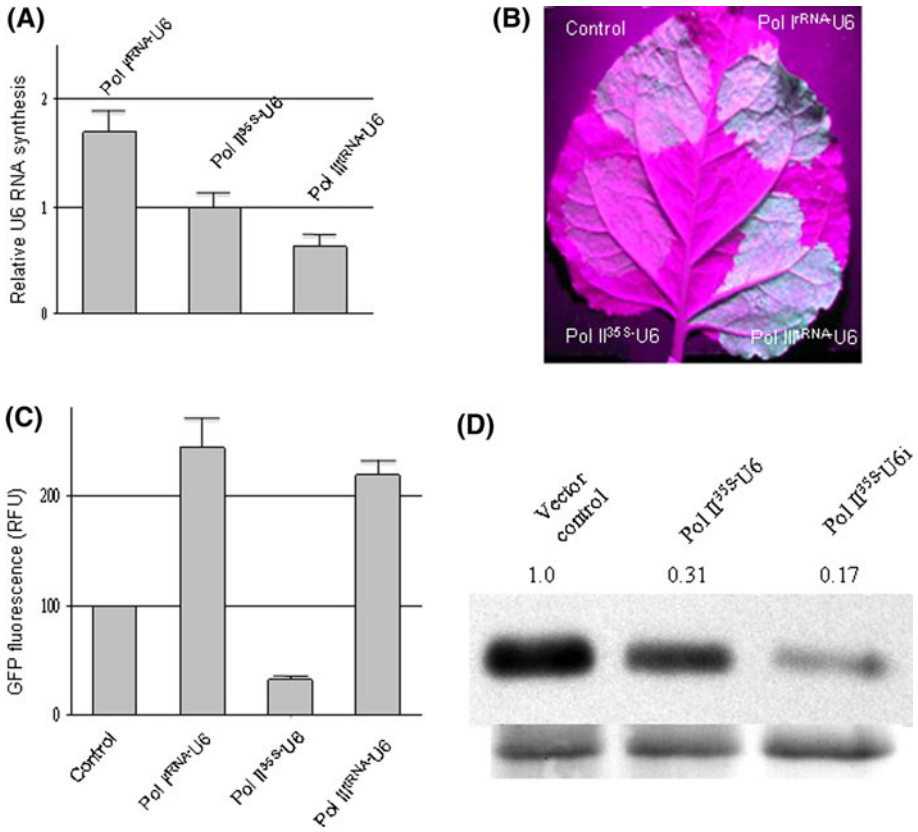


Fig. 6 Pol II-directed U6 snRNA suppresses the accumulation of GFP and BiP. (**a**) Relative quantity of U6 snRNA synthesised under the control of Pol I^{rRNA}-, Pol II^{35S}-, or Pol III^{tRNA}-dependent promoters determined by real-time PCR. (**b**, **c**) Visual detection and fluorimetric analysis of GFP expression in leaf sectors 3 days after co-agroinjection with GFP-expressing binary vector and vectors encoding U6 snRNAs under the control of Pol I^{rRNA}-, Pol II^{35S}-, or Pol III^{tRNA}-dependent promoters. Fluorescence measurements are presented in relative light units with the maximal GFP fluorescence, which was observed in co-agroinjection with empty pBin19, assigned a value of 100 (control). (**d**) Western blot and densitometry analyses of BiP in leaves agroinjected with Pol II^{35S}-$(GAAA)_{16}$ or Pol III^{tRNA}-$(GAAA)_{16}$. Numbers indicate the relative amounts of BiP as determined by band densitometry. Top bands, BiP; bottom bands, RuBisCO (gel loading control)

efficiency of the TMV vectors (Marillonnet et al. 2005), decreased its sensitivity to sncRNA (Fig. 7f).

Collectively, this suggests that competition between Pol II-directed sncRNA and mRNA for the nuclear export machinery results in suppression of the host cell antiviral response.

Pol II-directed short coding RNAs suppress host mRNA export and enhance TMV RNA replication

The idea that Pol II^{35S}-derived short RNAs suppress mRNA export by competing for the export machinery extends to cases of short mRNAs encoding, for example, pathogenesis related (PR) peptides such as proteinase inhibitors (PR-6 family), plant defensins (PR-12 family), thionins (PR-13 family) and lipid transfer proteins (PR-14 family) (for review see Sels et al. 2008). To support our model we used two scRNAs as competitors for GFP mRNA export (Fig. 8a): (1) the *N. benthamiana* antimicrobial peptide thionin DNA sequence with (802 nt) and without an intron (318 nt) (for review see Pelegrini and Franco 2005) and (2) an artificial 117-nt short sequence encoding Elastin-Like (for review see MacEwan and Chilkoti 2010) peptide element tandems with FLAG sequence (ELF). Like Pol II^{35S}-$(GAAA)_{16}$, co-agroinjection of Pol II^{35S}-ELF, resulted in a dramatic suppression of *GFP* mRNA expression in agroinjected leaves (Fig. 8b). Co-agroinjection of the 35S-based thionin gene with or without the intron also suppressed GFP production but to a lesser degree than Pol II^{35S}-ELF. In agreement with our model, both types of scRNAs (ELF and thionin genes) increased the cytoplasmic reproduction of TMV RNA (Fig. 8c).

We concluded that the overproduction of Pol II-directed sncRNAs and scRNAs in the nucleus results in the suppression of host mRNA export accompanied by the overproduction of viral RNA in the plant cytoplasm.

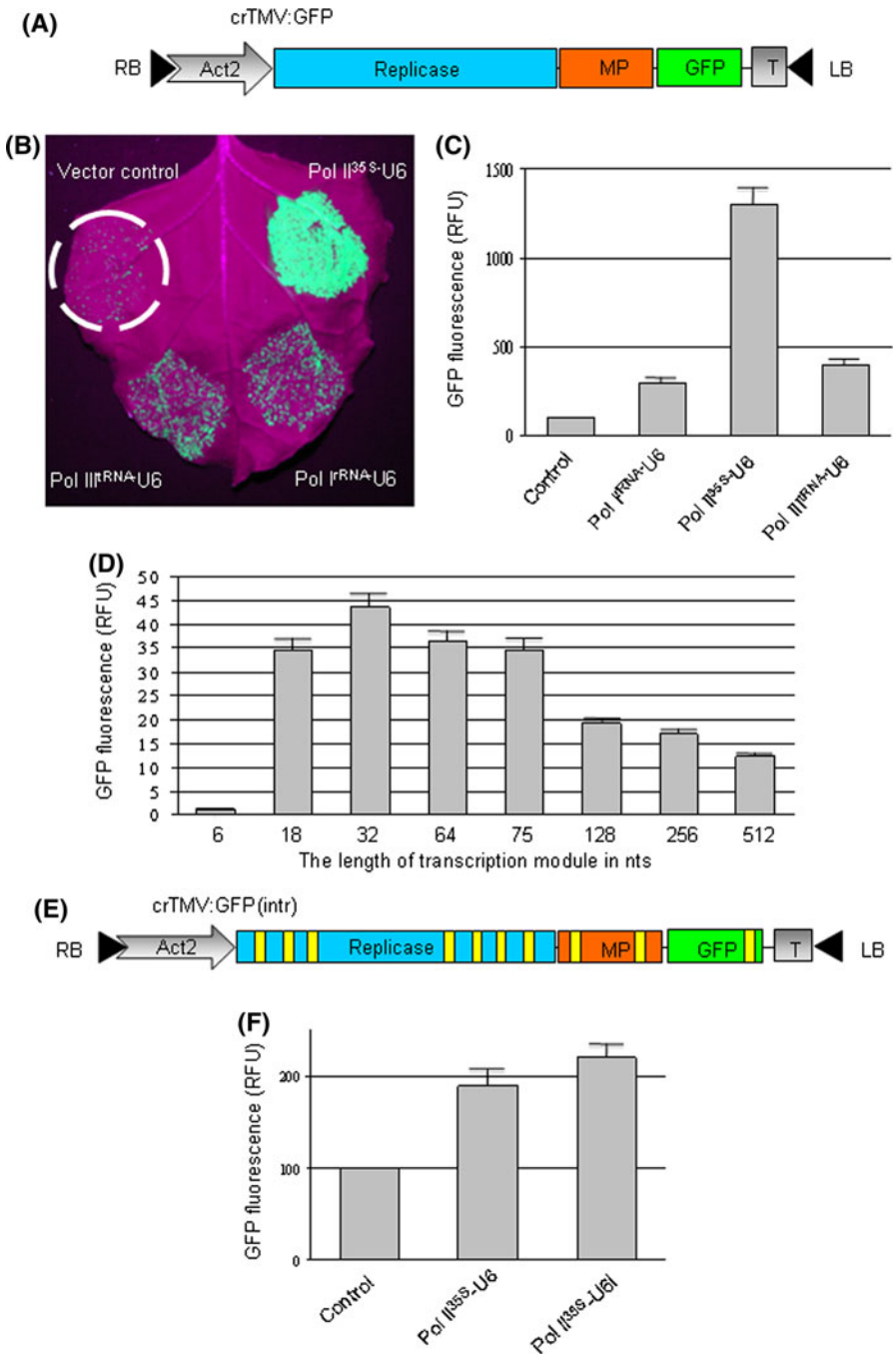


Fig. 7 Pol II-directed sncRNA enhances crTMV:GFP vector reproduction. (**a**) Schematic structure of the crTMV-based vector, crTMV:GFP, which contains the following genetic elements: LB and RB, left and right T-DNA borders, respectively; Act2, *Arabidopsis thaliana* Act2 promoter; Replicase, RNA-dependent RNA polymerase gene of crTMV; MP, movement protein gene; GFP; T, terminator of the Agrobacterium nopaline synthase gene. (**b**, **c**) Visual detection and fluorimetric analysis of GFP accumulation in leaves 3 days after co-agroinjection with crTMV:GFP and vectors encoding U6 RNAs under the control of Pol I^{rRNA}-, Pol II^{35S}- and Pol III^{tRNA}-dependent promoters. Fluorescence measurements are presented in relative light units with the maximal GFP fluorescence, which was observed in co-agroinjection with empty pBin19, assigned a value of 100 (control). Data were corrected for autofluorescence and represent 5-8 independent experiments with the standard *error bars* indicated. (**d**) Enhancement of viral reproduction by Pol II^{35S}-derived sncRNA depends on the transcription unit length. Fluorimetric analysis of GFP accumulation in leaves co-agroinjected with crTMV:GFP and Pol II^{35S}-based binary vectors encoding sncRNAs of different lengths. Fluorescence measurements are presented in relative light units with the maximal GFP fluorescence, which was observed in co-agroinjection with empty pBin19, assigned a value of 1.0 (control). The GAAA sequence module was used to construct transcription units with lengths of 6, 18, 32, 64, 128, 256, and 512 nucleotides. The 75-nt sncRNA is Pol II^{35S} $tRNA^{tyr}$. (**e**) Schematic structure of crTMV:GFP(intr) containing introns (indicated by yellow boxes) in viral genes. (F) Fluorimetric analysis of GFP accumulation in leaves co-agroinjected with TMV:GFP(intr) and Pol II^{35S}-U6 or Pol II^{35S}-U6i snRNA vectors

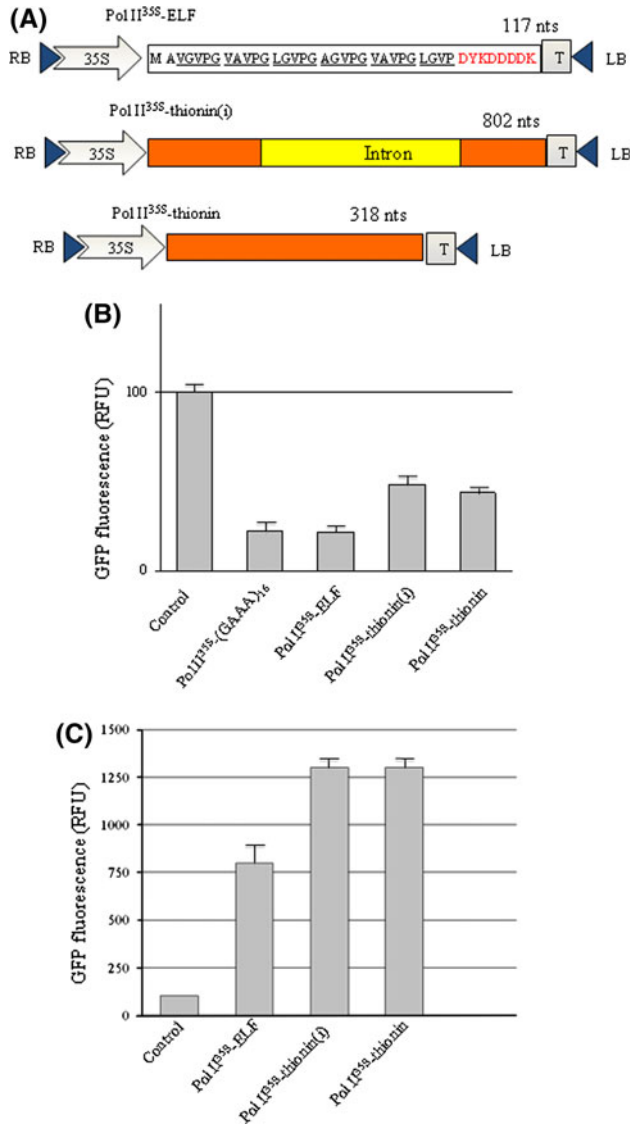


Fig. 8 Short mRNAs suppress the accumulation of GFP and enhances TMV:GFP vector reproduction. (**a**) Schematic structure of the Pol II^{35S}-ELF, Pol II^{35S}-thionin(i) and intron-less Pol II^{35S}-thionin vectors. The DNA length is shown. Elastin sequence elements are underlined. The FLAG sequence is shown in *red*. (**b**) Fluorimetric analysis of GFP expression in leaf sectors 3 days after co-agroinjection with GFP-expressing binary vector and vectors encoding $(GAAA)_{16}$, ELF and thionin. Fluorescence measurements are presented in relative light units with the maximal GFP fluorescence, which was observed in co-agroinjection with empty pBin19, assigned a value of 100 (control). (**c**) Fluorimetric analysis of GFP accumulation in leaves co-agroinjected with crTMV:GFP and vectors encoding short mRNAs. Fluorescence measurements are presented in relative light units with the maximal GFP fluorescence, which was observed in co-agroinjection with empty pBin19, assigned a value of 100 (control). Data represent five independent experiments with the standard *error bars* indicated

Discussion

Pols I, III and II are concentrated in distinct nuclear compartments in eukaryotic cells: the nucleolus (Pols I, III) (for review see Boisvert et al. 2007) and transcription factories (Pol II) (for review see Bartlett et al. 2006). It is generally accepted that Pol activity is spatially separated to increase the transcription efficiency and improve the regulation of ribosome maturation in the nucleolus and mRNA processing in transcription factories (see for example, Faro-Trindade and Cook 2006). However, recent studies of miRNA and siRNA biogenesis in nucleoli-associated Cajal bodies (for review see Pontes and Pikaard 2008) and accumulated evidence of nucleolus exploitation by many plant and animal viruses (Hiscox 2007) suggest spatial separation of the Pols reduces the competition between Pol I- or III- and Pol II-mediated macromolecule export into the cytoplasm.

In an earlier study using microinjection of Xenopus oocytes, a tRNA, a U snRNA, and an mRNA competitively inhibited their own export at concentrations at which they had no effect on the export of heterologous RNAs (Jarmolowski et al. 1994). Here we tested this idea using *Agrobacterium tumefaciens* to co-deliver Pol I, II and III promoter-based vectors into the plant nucleus to allow transcription of sncRNAs and GFP mRNA. This method provides synchronous gene expression because *Agrobacterium* can simultaneously infect at least 96% of the cells of injected leaves (Marillonnet et al. 2005).

A technique based on the binding of the coat protein of the MS2 bacteriophage (CP_{MS2}) to a 19-base hairpin (MS2 hairpin) was applied (Grünwald and Singer 2010; Mor et al. 2010; Zhang and Simon 2003) to visualise RNAs exported into the cytoplasm. This MS2 CP-based technique identified the sncRNAs under the control of CaMV 35S (Pol II^{35S}), rRNA (Pol I^{rRNA}) or $tRNA^{Tyr}$ (Pol $III^{tRNATyr}$) promoters in the plant cytoplasm (Fig. 1). We showed also that, in contrast to Pol I- and III-derived sncRNAs, all tested Pol II-sncRNAs (CaMV 35S-mediated U6, tRNA, or artificial RNAs) decreased the expression of *GFP* (Fig. 2) and host mRNA (BiP) (Fig. 6).

The level of competition depended on the splicing of Pol II-generated RNAs (Fig. 3), which is in accordance with a recent study on the dynamics of single mRNP nucleocytoplasmic transport and export through the nuclear pores of living cells (Mor et al. 2010).

Competition for the export machinery can explain stress-induced short mRNAs. We showed that an artificial 117-nt short sequence encoding ELF efficiently decreased the expression of GFP (Fig. 8b). A similar result was obtained with the *N. benthamiana* thionin (defensin) gene with or without its intron (Fig. 8b). Competition between different mRNAs may occur for the common nuclear export machinery and transport factors, as was recently suggested (Kimura et al. 2009).

Because Pol II^{35S}-derived sncRNAs and scRNAs can interfere with protective antivirus mRNA and miRNA for

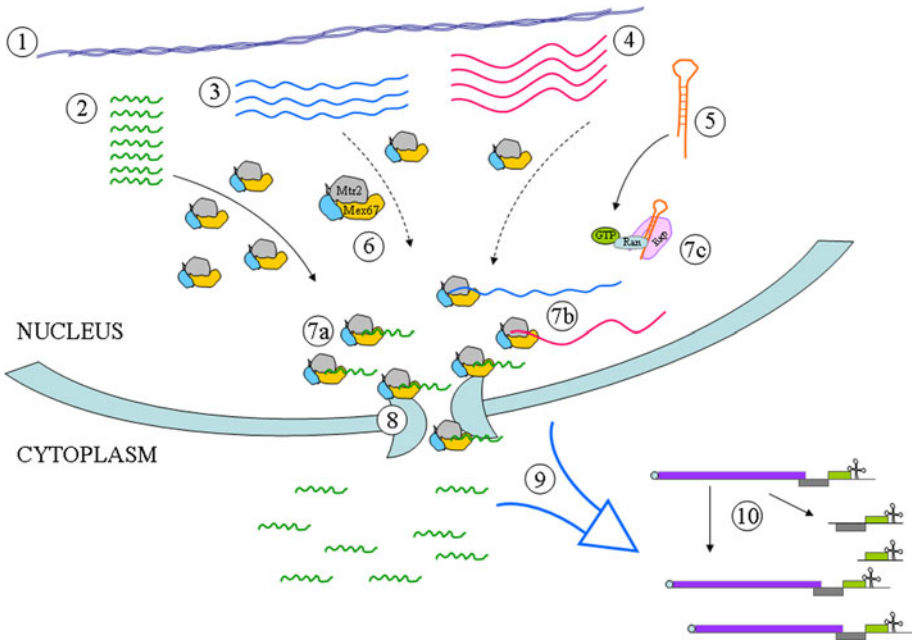


Fig. 9 Schematic representation of competition between Pol II-synthesised short (stress-induced) RNAs and all other cellular mRNAs. *1* DNA; *2* large amount of intensively synthesised short stress-induced RNAs (coding or non-coding); *3* mRNAs encoding RNAi factors; *4* mRNAs encoding cellular stress-induced proteins; *5* virus-induced miRNAs; *6* complex of proteins for mRNA export from the nucleus (including the Mtr2-Mex67 heterodimer and various adaptors); *7a* small RNPs; *7b* large mRNPs; *7c* pre-miRNPs (containing Ran-GTP and exportin); *8* nuclear pore complex; *9,10* the impeded nuclear export of cellular defensive RNAs (*9*) leads to intensive viral reproduction (*10*)

nuclear export, they may create conditions for enhanced viral replication in the cytoplasm. To test this hypothesis, we exploited binary vectors directed to the synthesis of genomic TMV and PVX RNA in the host nucleus. In contrast to natural infection where viral RNA directly enters the cytoplasm of a negligible number of cells after wounding the leaf, agroinjection allows the simultaneous infection of the nuclei of almost all leaf cells. Co-agroinjection of vectors encoding short RNA and viral RNA resulted in mutual competition and competition with protective antiviral mRNAs and miRNAs. Nevertheless, we assumed that decreased viral RNA export into the cytoplasm would be accompanied by enhanced virus replication and stabilisation of viral RNA in the cytoplasm lacking silencing factors. Our results agreed with this hypothesis (Figs. 7 and 8), and the overproduction of Pol II-directed sncRNAs and scRNAs in the nucleus results in the overproduction of viral RNA.

Recent advances in the study of mRNA export have shown that Pol II-directed transcription induces chromosomal movement, tight association with the nuclear pore complex (NPC) and close apposition of promoter and terminator regions (Moore and Proudfoot 2009). We believe that the synthesis of sncRNA or scRNAs may block the access of mRNA to the NPC. Our model of competition between Pol II-synthesised short RNAs and all other cellular mRNAs (Fig. 9) suggests that a large amount of coding or non-coding intensively synthesised short stress-induced RNAs enter the transcription export (TREX) complex and recruit Mex67-Mtr2 to form small RNPs (for review see Stewart 2010). Then, small RNPs and pre-miRNPs (containing Ran-GTP and exportin) (for review see Stewart 2009) interact with the NPC and compete for NPC docking with large mRNPs containing other cellular mRNAs including those encoding silencing factors. Finally, small RNPs with short coding or non-coding RNAs prevent large mRNPs from nuclear export. This model agrees with a study describing a three-step transport mechanism of mRNP export involving nucleoplasmic docking, rapid translocation through the central channel and cytoplasmic release (Grünwald and Singer 2010).

To analyse the biological significance of these results regarding plant viruses, it is important to consider that the host plant reacts to viral invasion (mechanical inoculation or vector attack, for example) by inducing sncRNA and stress proteins. Mechanical wounding and therefore stress induction is inevitable during viral infection especially during the initial steps. Interestingly, a correlation exists between the accelerated rate of evolution of plant viruses and the beginning of agriculture on Earth, as handling plants increases the likelihood of viral invasion due to plant wounding and virus contamination (Gibbs et al. 2010).

Acknowledgments We thank Drs. C. S. Pikaard (at Washington University St. Louis), T. Okuno (Kyoto University), A. E. Simon (University of Maryland, College Park) and H. Beier (University of Würzburg) for generously providing the Pol I promoter containing plasmid pBor2, pBICmiR171prec, GNC encoding plasmid, and plasmid pNtY1 encoding *Nicotiana rustica* pre-tRNA^{Tyr}, respectively. We also thank members of the MSU Department of Virology for helpful discussions and technical assistance. This work was partly supported by the Russian Foundation for Basic Research (grants 08-04-00106 and 08-04-12073) and Icon Genetics GmbH. The work in the VC laboratory is supported by grants from NIH, NSF, NRI USDA CSREE, BARD, and BSF.

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