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Stability of Plant mRNAs Depends on the Length of the 3′-Untranslated Region[1]

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Abstract—Eukaryotic mRNAs that prematurely terminate translation are recognized and degraded by nonsense mediated decay (NMD). This degradation pathway is well studied in animal and yeast cells. The data available imply that NMD also takes place in plants. However, the molecular mechanism of recognition and degradation of plant RNAs containing premature terminator codon (PTC) is not known. Here we report that in plant cells this mechanism involves the recognition of the sizes of the 3′-untranslated regions (3′UTR). Plant 3′UTRs longer than 300 nucleotides induce mRNA instability. Contrary to mammalian and yeast cells, this destabilization does not depend on the presence of any specific sequences downstream of the terminator codon. Unlike nuclear-produced mRNAs, plant virus vector long 3′UTR-containing RNAs, which are synthesized directly in the cytoplasm, are stable and translated efficiently. This shows that RNAs produced in the cytoplasm by viral RNA-dependent RNA polymerase are able to avoid the proposed mechanism.

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Key words: nonsense mediated decay, RNA stability, agroinjection

Nonsense mediated decay (NMD) is a mechanism that eliminates aberrant mRNAs produced due to abnormalities in genes structure and expression. NMD prevents the production of prematurely terminated proteins that could function in dominant-negative or other deleterious mechanisms [1]. Various human genetic diseases are associated with mutant genes containing frameshift or nonsense mutations that generate premature-termination (nonsense) codons [1-3]. Functional NMD is crucial under these conditions.

The mechanisms of NMD have been extensively studied in mammals and yeasts. In mammals, NMD involves the exon junction complex (EJC) of proteins being deposited as a consequence of pre-mRNA splicing. The deposition takes place upstream of exon–exon junctions generated after the intron removal. It is widely accepted that premature termination codons (PTC) combined with an intron located further than 50-55 nucleotides (nt) downstream generally elicit NMD ([4-6], for review see [7-9]). A current model of NMD suggests that the decision whether mRNA enters the NMD pathway or joins the pool of translated molecules takes place at the very first round of mRNA translation. This pioneer event is mediated by a cap-binding complex (CBC), which binds to the cap structure of nascent pre-mRNAs in the nucleus. CBC consists of CBP80–CBP20 heterodimer associated with the cap and remains associated with mRNAs during their export to the cytoplasm.

Abbreviations: CaMV) cauliflower mosaic virus; CBC) cap binding complex; CBP) cap binding protein; DSE) downstream element; eIF4E) eukaryotic translation initiation factor 4E; EJC) exon junction complex; IRES) internal ribosome entry site; NMD) nonsense mediated decay; NOM) natural open reading frame marker; PTC) premature termination codon; PVX) potato virus X; sg) subgenomic; UTR) untranslated region.

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[1] When this manuscript was submitted, an article by Kertesz et al. (2006) was published reporting similar results on NMD mechanism and realization in plants. The two studies were carried out independently, and while strengthening the results of each other they provide complementary insights into the NMD phenomenon. Preliminary results of our study were presented as T. V. Komarova et al., "Recognition of nonsense RNA in plants", XV FESPB Congress, Lyon, France, 2006, July 17-21, GEN02-13, p. 108, and as M. V. Skulachev et al., "Internal termination of translation causes nonsense-mediated decay of mRNA in plants", The 8th International Engelhardt Conference on Molecular Biology, Moscow Region, Russia, 2006, August 19-24, p. 32.

Once mRNA passes the NMD checkpoint successfully, its CBC is replaced by eukaryotic translation initiation factor 4E (eIF4E) and the mRNA is directed to a steady-state translation. However, aberrant mRNA molecules containing PTC are recognized and subjected to decapping to induce their 5′-3′ degradation. The recognition of PTC is due to the fact that the EJC is located downstream of the termination codon and thus cannot be removed from mRNA by a translating ribosome. If the EJC remains bound to mRNA due to premature termination of translation, then the mRNA is subjected to NMD. It is accepted that NMD mechanisms are generally similar in mammalian and yeast cells with one significant exception – yeasts utilize a special consensus sequence instead of exon–exon junctions to mark the natural coding region of mRNA. This sequence termed downstream element (DSE) of instability was identified as 5′-TGCTGATGCTTTCTCTGCTGATGC-3′ (a less stringent version of this motif 5′-TGYYGATGYYYYY-3′) [10]. With the exception mentioned above, yeasts and mammals share the general principle of NMD functioning based on the special mRNA motifs that are recognized by specialized protein complexes. Such motifs can be termed natural ORF markers (NOMs). When premature termination of translation fails to remove all complexes from NOMs, mRNAs are recognized as abnormal and NMD is triggered.

The mechanism of NMD in plants is still poorly understood. Nonsense or frameshift mutations were shown to drastically reduce the abundance of mRNAs encoding. This is demonstrated by the experiments with soybean Kunitz trypsin inhibitor (Kti3) [11], bean phytohemagglutinin (PHA) [12, 13], tobacco ferredoxin-1 (Fed-1) [14, 15], and rice *waxy* gene products [16].

The phenomenon of plant mRNA destabilization was shown to be associated with translation and dependent on the position of nonsense codon within the plant gene. A PTC located less than 170 nt upstream of a natural termination codon does not elicit NMD, while the degradation of mRNA occurs in case PTC is inserted further than 300 nt upstream of a normal terminator [13, 15]. It was suggested that this effect might be mediated by a hypothetical NOM of plant mRNAs [15]. Apparently, if this was the case, the plant-specific NOMs are different from their mammalian and yeast analogs since NMD in plants is independent of mRNA introns [14] and no sequences similar to yeast DSE were found.

The idea of the existence of NMD in plants is supported by the fact that a number of genes with significant homology to mammalian NMD factors were found in plant genomes. These include genes homologous to CBP80 (GeneID: 815840), CBP20 (GeneID: 834443), Upf1 (GeneBank AC: AF484122), Upf2 (GeneID: 3062583), Upf3 (GeneID: 840295), Y14 (GeneID: 841576), Dcp1p (GeneID: 837357). Moreover, it was recently shown [17] that a mutation in a gene homologous to Upf3 suppresses degradation of aberrantly spliced RNA with premature termination codons in *Arabidopsis thaliana*. Also it was also reported that the null mutant of putative UPF 1 is lethal [18].

The present work focuses on studying general principles of NMD in plants. In particular, mRNA features recognized by plant RNA quality control mechanism are examined.

MATERIALS AND METHODS

Plasmid construction. 35S promoter with S1 leader, synthetic GFP gene [19], and 35S terminator were inserted in sequential order into pCambia1300 vector yielding 35S-GFP construct (also named GFP-40). Stop711, Stop360, and Stop15 constructs were obtained via cloning of PCR product containing the GFP gene with corresponding stop codons into 35S-GFP replacing the GFP gene. PCR products were obtained using the following primers: "GFP-Stop2-p", "GFP-Stop119-p", "GFP-Stop119-m", and "GFP-Stop234-m". PCR fragment (obtained from primers "IREScpKpn–" and "GFPDir+") containing a copy of $IRES^{Cr}_{CP,148}$ [20] flanked with *Kpn*I sites was inserted in direct and reverse orientation into the *Kpn*I site downstream of GFP yielding GFP-180a, b constructs, respectively. To obtain GFP-380 construct, the same PCR product was inserted twice (in direct orientation). GFP-340a, b contained 35S promoter with S1 leader, GFP gene, 5′-proximal 340 bp of RFP gene (for GFP-340a in direct and for GFP-340b in reverse orientation), and 35S terminator inserted into pCambia 1300. In 35S-GFP-1010 construct PVX CP gene fused to $IRES^{Cr}_{CP,148}$ (both in reverse orientation) was cloned downstream of the GFP gene.

35S-GFP-RFP construct was made by cloning of RFP gene into 35S-GFP pCambia-based plasmid. That fragment was obtained by PCR using primers "RFP(+)" and "RFP(–)". The GFP gene without stop codon was obtained by PCR using "GFP-Nco+" and "GFPw/o stop" primers. GFP in 35S-GFP-RFP plasmid was substituted by this PCR product yielding 35S-GFPRFPf construct.

A stable hairpin was cloned upstream of the GFP gene in 35S-GFP-RFP construct as described in [20]. $IRES^{Cr}_{CP,148}$ was inserted in 35S-GFP-RFP construct between GFP and RFP genes using *Bam*HI-*Nco*I sites yielding 35S-GFP-IRES-RFP plasmid. Note that RFP start ATG codon was restored in this construct.

Viral vectors were made in several cloning steps and were based on PVXdt-GFP plasmid [21]. The resulting construct, PVX:GFP-RFP, contained, in sequential order, 35S promoter, RNA-dependent RNA-polymerase gene from PVX (with its original 5′-UTR), subgenomic promoter, GFP gene, RFP gene without start codon, 3′-UTR from PVX genome, and 35S terminator. PVX: GFPRFPf

contained GFP without stop codon and RFP without start codon resulting in fused GFPRFPf gene. To obtain these constructs fragments GFP-RFP and GFPRFPf were inserted into PVXdt-GFP [21] replacing the GFP gene.

Primer sequences:
IREScpKpn–, ATATGGTACCTTTCTTCTTTCAAATTAAACGAATC;
GFPDir+, ACATGGTCCTGCTGGATGTCG;
GFPw/o stop, TTTTGGATCCTATTCTTGCGGGCCCTCGCTTGTACAGCTCGTCCATGCC;
GFPNco+, ATATCCATGGTGAGCAAGGGCGAGGA;
RFP(+), TTTTGGATCCATAAGAACGGGGCCCATCGCCTCCTCCGAGGACGTC;
RFP(–), TTTTTCTAGATTAGGCGCCGGTGGAGTGGCG;
GFP-Stop2-p, ATATCCATGGTGTAAGGGCGAGGAGCT;
GFP-Stop119-p, TAACTGGTGAACCGCATCGAGCT;
GFP-Stop119-m, GGTTCACCAGTTAGTCGCCCTCGAACTTCA;
GFP-Stop234-m, ACTTGTACAGCTCGTTACATGCCGTGAGTGAT.

Agroinjection. A 500-μl sample of overnight *Agrobacterium tumefaciens* (GV-3101 strain) culture was precipitated at 2300*g* for 5 min, and the pellet was resuspended in solution containing 10 mM MES (pH 5.5) and 10 mM $MgCl_2$ to final A_{600} 0.2 (if the mixture of different strains was used, then final A_{600} of each culture was 0.2). Leaves of greenhouse grown *Nicotiana benthamiana* plants were injected with this suspension using a syringe without needle.

Protein extraction from agroinjected spots and SDS-PAGE analysis. On the third day after infection, injected spots (~10 mg) were ground in the presence of celite in 40 μl of extraction buffer (60% glycerol, 5 mM β-mercaptoethanol, 10% SDS, 250 mM Tris-HCl, pH 6.8) for total protein extraction. Protein extracts were fractionated using SDS-PAGE and examined by Western-blot analysis.

Western-blot analysis. Fractionated proteins were transferred to Hybond-P PVDF membrane (Amersham), blocked with 5% skim milk solution, incubated with monoclonal anti-GFP antibodies (Roche, Switzerland), and then GFP was detected by an ECL Western-blotting detection kit (Amersham, England) using anti-mouse peroxidase conjugate (Sigma, USA). After Western-blotting, membranes were stained with Amido Black solution (0.1% Amido Black (Reanal, Hungary), 25% isopropanol, 10% acetic acid) for evaluation of total protein amount loaded in each lane (loading control).

Total RNA extraction and Northern-blot analysis. Total RNA was extracted from leaves on the 2nd or 3rd (in case of viral vectors) day after injection according to [22], fractionated in 1.5% agarose gel under denaturing conditions (10% formaldehyde), and transferred to a Hybond-XL nylon membrane (Amersham). Membranes were probed with a denatured single-stranded anti-GFP DNA fragment labeled with [α-^{32}P]dATP and then washed and exposed with X-ray film (Retina).

Prior to hybridization, membranes were stained with methylene blue solution (0.5 M sodium acetate, pH 5.2, 0.04% methylene blue) to estimate the amount of loaded RNA.

RESULTS AND DISCUSSION

Plant cells degrade foreign nonsense mRNAs via NMD. Plant NMD was previously studied using endogenous plant genes. Most of these studies were not aimed directly at the investigation of the NMD phenomenon. Instead, they were focused on the identification of the PTC-containing mutant plant gene and demonstration of the corresponding mRNA degradation [16, 23].

The first question we addressed is whether the PTC-containing RNAs of non-plant origin can be subjected to NMD in plant cells, i.e. if foreign aberrant mRNAs are degraded in plants. Elucidation of this question was particularly important for identification of the hypothetical plant NOMs.

Agrobacterium-based injections of *Nicotiana benthamiana* leaves were used for transient expression of artificial mRNAs. Three constructs (Fig. 1a) based on the jellyfish green fluorescent protein (GFP) gene were used in agroinjections (Stop711, Stop360, and Stop15). These mutant constructs contained a premature terminator codon upstream of the natural GFP stop: 711 nt (third codon of the gene), 360 nt (119th codon), and 15nt (234th codon), respectively.

In the case when foreign and endogenous "nonsense" mRNAs (i.e. mRNAs containing premature nonsense codon) are recognized in plants by the same mechanism, Stop15 mRNA is expected to accumulate at the levels of non-mutated GFP mRNA, while Stop360 and Stop711 mRNAs are expected to be degraded by NMD [13, 14]. Our results (Fig. 1b) were consistent with the above hypothesis: accumulation of GFP mRNA with the PTC located further than 300 nt upstream of the natural stop signal was drastically inhibited. At the same time, the construct with PTC located close (15 nt) to the natural terminator was not subjected to NMD, whereas the PTC located immediately downstream of GFP start codon (Stop711) exhibited a moderate destabilizing effect compared to the PTC in the middle of the gene.

This experiment provides evidence that similar mechanisms are used by plant cells for the recognition of endogenous and foreign PTC-containing mRNAs. Therefore, these data do not support the hypothesis of the existence of specific NOMs in plant RNAs (since it is unlikely that jellyfish GFP gene contains plant NOMs).

It was shown in yeasts [24] and animals [25] that NMD can be triggered by elongation of 3′UTR causing termination of translation to occur distantly from the 3′-

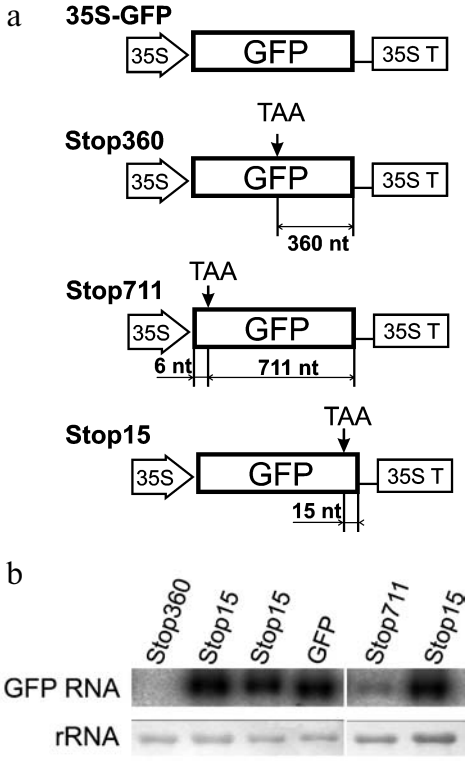

Fig. 1. Accumulation of artificial PTC-containing mRNAs in agroinjected plant leaves. a) Schematic representation of constructs. 35S and 35S-T boxes correspond to 35S promoter and terminator of CaMV, respectively. Distances (in nt) between PTC and natural GFP stop are indicated for constructs Stop360, Stop15, and Stop711. b) Northern-blot analysis of RNAs expressed in *N. benthamiana* leaves agroinjected with the constructs. mRNAs were detected using anti-GFP ^{32}P-labeled probe. Methylene blue stained rRNA on lower panel represents loading control for each lane.

end of the mRNA. The authors suggested that exceeding a certain distance between the terminator codon and the polyA tail serves as a signal for RNA destabilization. Obviously, such mechanism does not require any NOM. Therefore, a hypothesis can be proposed that similar mechanism can be utilized for recognition of PTC in plants.

mRNAs with long 3′UTRs are expressed poorly and are infrequent in plant cells. To test the above hypothesis we artificially elongated 3′UTR of 35S-GFP construct used in the previous experiment. As a result, a set of model constructs expressing mRNAs with extended 3′UTR was made. These constructs were obtained by cloning of different DNA fragments and their reverse complement sequences downstream of GFP natural stop codon (Fig. 2a). The constructs were transiently expressed in *N. benthamiana* leaves by agroinjection. The levels of mRNA accumulation driven by each construct were compared with that of 35S-GFP (marked as GFP-40 to indicate the 40-nt 3′UTR of corresponding mRNA) using Northern-blot analysis. Relative mRNA abundance was calculated by densitometry of the Northern-blots of at least three independent experiments for each construct (amount of GFP mRNA produced by the GFP-40 construct was taken as 100%). Results presented in Fig. 2b clearly demonstrate that all GFP mRNAs with 3′UTRs

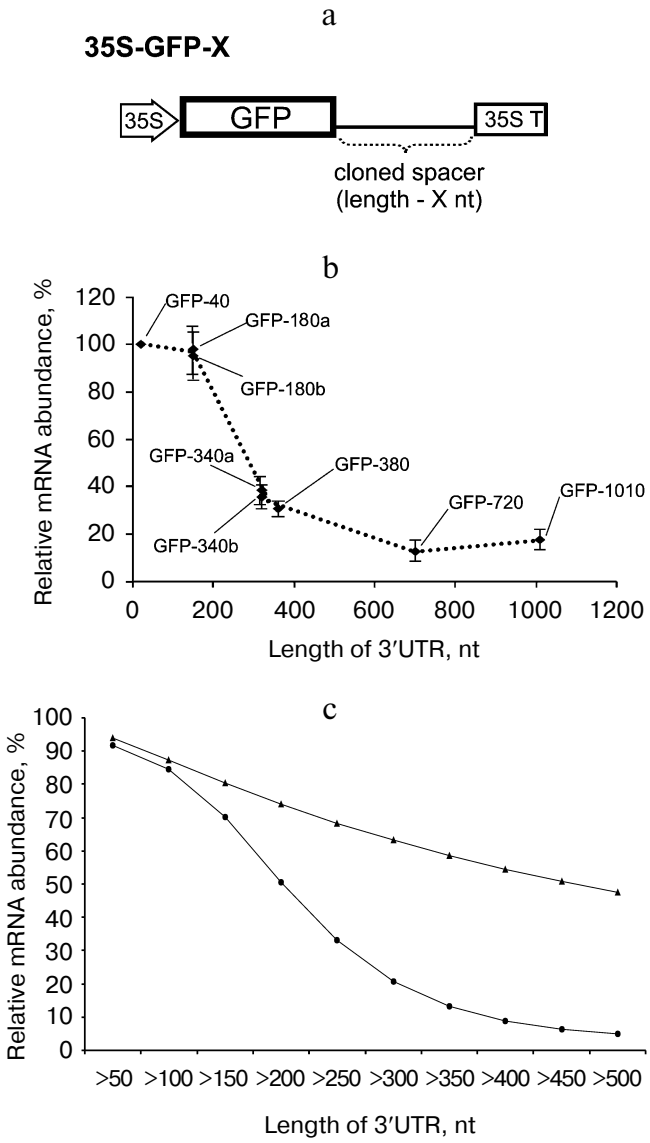

Fig. 2. Abundance of plant mRNA depends on the length of 3′UTR. a) Schematic representation of the model GFP-based constructs with different 3′UTRs (X represents approximate length of the 3′UTR). b) Dependence of the GFP mRNA accumulation on its 3′UTR length. Amount of accumulated RNA was calculated by densitometry of Northern-blot bands detected by hybridization with anti-GFP probe. Amount of RNA produced by monocistronic GFP-40 was taken as 100%. Numerals in the constructs names indicate the length of insert (cloned spacer) between GFP stop codon and polyadenylation signal. Each point on the diagram corresponds to average result of at least three independent experiments. The 95% probability intervals are presented. c) GeneBank database analysis. Each point corresponds to the percentage of mammalian (triangles) or plant (circles) mRNAs that have 3′UTR longer than the threshold indicated on X-axis.

exceeding 300 nt were accumulated poorly independently on the sequence of the spacer cloned.

Presuming that these results reflect some general principle of plant mRNA turnover, it suggests that plant mRNAs with long 3′UTR are unstable. Thus, mRNAs with long 3′UTRs are expected to be rare among plant mRNAs compared to yeasts and mammals.

To test this hypothesis, a series of GeneBank and UTResource (http://bighost.area.ba.cnr.it/BIG/UTRHome) databases were analyzed. The results of this study are summarized in Fig. 2c. Computer analysis of 78398 plant mRNAs and 95173 mammalian RNAs showed that, contrary to mammals, plants clearly avoid long 3′UTRs (each dot on the diagram indicates the percent of mRNAs containing 3′UTR longer than a certain number) (indicated on X-axis, Fig. 2c). The same analysis was performed for approximately 3000 human and 3000 plant genes with validated 3′UTR. The analysis produced similar result (data not shown).

Together, the results obtained by computer analysis (Fig. 2c) and experimental data with artificial mRNAs (Fig. 2b) indicate that the observed effect relates to basic features of plant mRNA structure and stability.

RNAs with extended 3′UTR are possibly subjected to NMD. The above experiments show that mRNAs with long 3′UTR are unstable and rare in plants. We suggested a hypothesis that these phenomena are associated with NMD due to recognition of such RNAs as "nonsense". However, the observed effects can be also explained by the inefficient transcription of the engineered constructs. Indeed, elongation of RNA 3′UTR inevitably leads to the elongation of the whole RNA that, in turn, could possibly decrease the efficiency of transcription. The following experiment was designed to test this possibility.

The construct 35-GFP-720 used in the previous experiment (Fig. 2) was made by cloning of the red fluorescent protein (RFP) gene downstream of the GFP gene. This construct is termed 35S-GFP-RFP (Fig. 3a). To obtain an appropriate transcription control construct (mRNA of the same size with fully translatable RFP ORF) the GFP stop codon was eliminated, creating a single fused ORF (construct 35S-GFPRFPf, Fig. 3a).

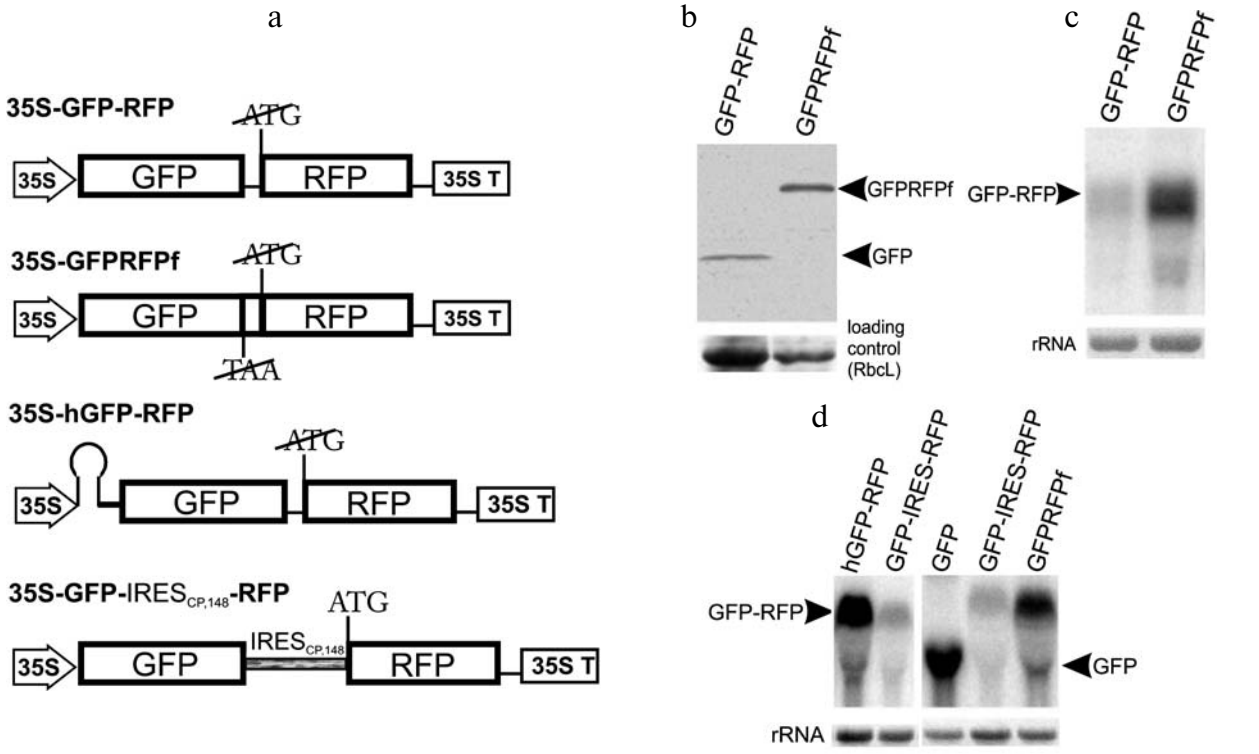

Fig. 3. Accumulation of mRNA with extended 3′UTR in plants. a) Schematic representation of constructs. Crossed out TAA in the construct 35S-GFPRFPf indicates that GFP stop codon is mutated and continuous fusion ORF is formed. Crossed out ATG indicates the absence of RFP start codon. Curve upstream of GFP in the hGFP-RFP construct represents inverted repeat that produces a stable stem-loop structure in mRNA, and IRES corresponds to $IRES^{CR}_{CP,148}$ from crucifer tobamovirus genome. b) Western-blot analysis of GFP and GFPRFP-fusion expression in *N. benthamiana* leaves agroinjected with 35S-GFP-RFP and 35S-GFPRFPf. Amount of total protein loaded in each lane can be estimated by the loading control on the lower panel. c) Northern-blot analysis of RNAs expressed in the same leaves. Anti-GFP radioactive probe was used. Amount of total RNA loaded in each lane is estimated by loading control on lower panel. Position of GFP-RFP RNA is indicated by arrow. d) Northern-blot analysis of RNA produced in *N. benthamiana* leaves agroinjected with 35S-hGFP-RFP, 35S-GPF-IRES-RFP, and 35S-GFP. Anti-GFP radioactive probe was used. Amount of total RNA loaded in each lane is estimated by loading control on lower panel. Position of GFP-RFP and GFP RNAs are indicated by arrows.

Both Western-blot and Northern-blot data (Figs. 3b and 3c, respectively) show that the control fusion 35S-GFPRFPf construct was expressed much more efficiently comparing to 35S-GFP-RFP in agroinjected *N. benthamiana* leaves. This result shows that elongation of GFP RNA by 680 nt does not inhibit its transcription in plant agroinjection assay and that a single stop codon in the middle of the mRNA is responsible for its poor expression.

An important difference must be noted between experiments with GFP carrying PTC (Fig. 1) and GFP with extended 3′UTR (Figs. 2 and 3). While in the first case unstable RNAs contained PTC and thus may be regarded as "nonsense" RNA, in the latter experiments elongation of the 3′UTR caused degradation of mRNA containing natural GFP terminator. As no premature termination of translation took place in this case, one cannot exclude that different mechanisms are responsible for PTC-derived destabilization of mRNA and poor accumulation of mRNAs with long 3′UTR. However, all our experiments (and previous studies of plant NMD by van Hoof and Green [13]) clearly demonstrate the dependence of RNA accumulation on the distance between terminator codon and RNA 3′-end. In all cases, for both premature and natural stop codon-containing RNAs, the RNAs with the 3′UTRs longer than 300 nt were poorly accumulated. It must be also noted that natural GFP stop codon in GFP-RFP construct can be regarded as PTC of fusion GFPRFPf gene. Therefore, the last experiment reveals a common mechanism of PTC- and natural stop-dependent mRNA degradation pathways.

It is accepted that "nonsense" RNA recognition and NMD in all eukaryotes (including plants [14]) depends strictly on translation. Here we tested whether destabilization of mRNA with extended 3′UTR also can be prevented by translational arrest of the mRNA.

To abolish the translation, a hairpin structure comprising 60-nt GC-rich inverted repeats was inserted at the 5′-end of unstable GFP-RFP mRNA (Fig. 3a). This hairpin was previously shown to completely inhibit translation initiation *in vitro* [26]. Another construct was made on the basis of 35S-GFP-RFP. An internal ribosome entry site (IRES) from the crucifer tobamovirus genome was inserted upstream of the RFP gene. This IRES was previously shown to be active in plants (both *in vitro* and *in vivo*), but its activity is low compared to usual cap-dependent translation [20, 27-29]. RFP ORF in the resulting 35S-GFP-IRES-RFP construct can be translated despite its 3′-proximal position downstream of GFP ORF (Fig. 3a).

The constructs were delivered into *N. benthamiana* leaves by agroinjection. Accumulation of corresponding mRNAs was detected by Northern-blot analysis (Fig. 3d). It was found that untranslatable hGFP-RFP mRNA was accumulated with efficiency comparable to that of the control GFPRFPf mRNA and much more efficiently than unstable GFP-RFP mRNA (Fig. 3d). These results indicate that translational arrest of hGFP-RFP mRNA containing extended 3′UTR rescued it from degradation. Consequently, translation in plant cells of mRNAs carrying extended 3′UTR is essential for their destabilization. It must be also noted that introduction of $IRES^{CR}_{CP,148}$ downstream of the GFP gene did not increase accumulation of GFP-RFP mRNA. This result reveals serious limitation for the use of bicistronic constructs in plant cells. It also confirms the existence of a common mechanism of

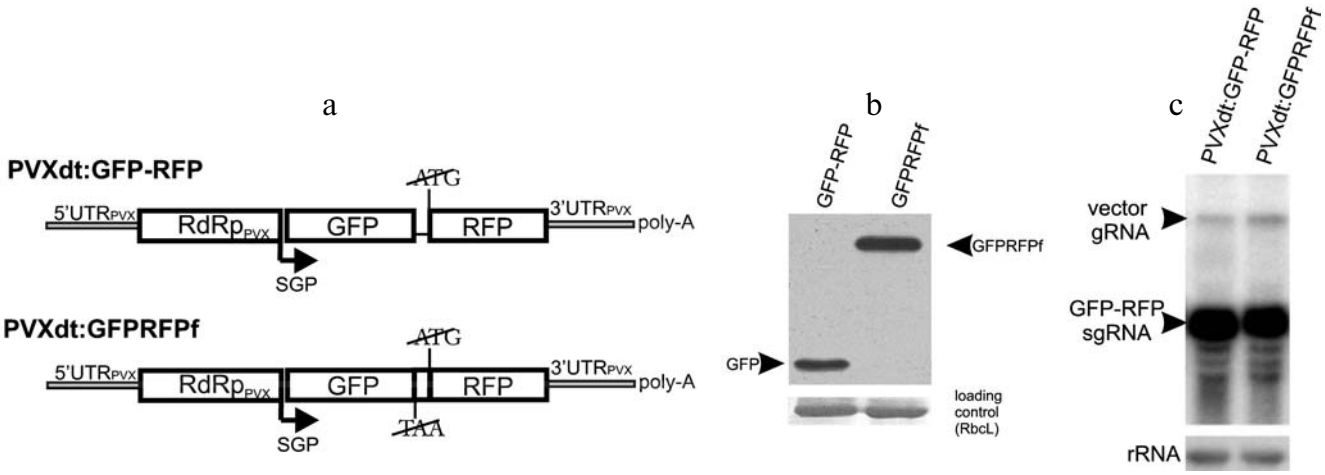

Fig. 4. Efficient expression of bicistronic constructs by RNA-virus based vector-replicon. a) Schematic representation of the vectors: SGP corresponds to PVX 25-kD gene subgenomic (sg) promoter; arrow indicates the direction of sgRNA synthesis. Two variants of vector were obtained: PVX:GFP-RFP carrying bicistronic insert downstream of indicated sg promoter and PVX:GFPRFPf carrying the fusion ORF. b) Western-blot analysis of GFP and GFPRFP-fusion protein expression in *N. benthamiana* leaves agroinjected with described constructs. Amount of total protein loaded in each lane can be estimated by loading control on lower panel. Position of GFP-RFP and GFP are indicated by arrows. c) Northern-blot analysis of RNAs expressed in *N. benthamiana* leaves agroinjected with these constructs. Anti-GFP radioactive probe was used. Amount of total RNA loaded in each lane estimated by loading control on lower panel. Positions of vector full-length genomic RNA (gRNA) and GFP-RFP sgRNA are indicated.

PTC- and natural stop-dependent mRNA degradation pathways.

Virus vector-produced subgenomic mRNAs with extended 3′UTR are stable in plant cells. According to our data, RNAs with long 3′UTR (including polycistronic mRNAs) are accumulated poorly in plants. This conclusion obviously contradicts the vast abundance of polycistronic mRNAs among plant RNA viruses. Apparently, plant viral RNAs (at least RNAs of viruses replicating in the cytoplasm; see below) escape degradation even when they contain long 3′UTR.

To study this phenomenon more closely, we tested virus-mediated expression and stability of GFP-RFP mRNA, which is recognized as "nonsense" RNA when transcribed under control of the 35S promoter in agroinjection experiments. Two constructs were created on the basis of the potato virus X (PVX) genome. PVX is an RNA plant virus with solely cytoplasmic life cycle. Its genome is represented by a molecule of genomic (+) RNA containing genes for RNA-dependent RNA-polymerase (RdRp), 25, 12, and 8 kD proteins involved in cell-to-cell movement of the virus, and the 25 kD coat protein. The 25- and 12-kD movement proteins and the coat protein are expressed via synthesis of separate subgenomic RNAs (sgRNAs).

GFP-RFP and GFPRFPf constructs were cloned into PVX-based vector-replicon PVXdt [21] (Fig. 4a) containing RdRp gene and subgenomic promoter responsible for synthesis of sgRNA. The PVX:GFP-RFP and PVX:GFPRFPf vectors replicated efficiently in the cytoplasm of the infected cell, producing GFP-RFP mRNA or GFPRFPf mRNA, respectively. It is noteworthy that these subgenomic transcripts were identical to GFP-RFP and GFPRFPf transcripts produced in the nucleus of *N. benthamiana* cells (see above).

Northern-blot analysis showed that the production efficiency of sgRNAs was similar between these vectors (Fig. 4c). This observation was also supported by the comparison of protein synthesis efficiencies (GFP and fusion GFPRFP protein, Fig. 4b). Thus, contrary to mRNA transcribed in the nucleus, mRNAs with extended 3′UTR appear not to be recognized as aberrant when expressed directly in the cytoplasm.

This phenomenon can be explained assuming that plants utilize the same general scheme of NMD as mammals, i.e., recognition of PTC occurring on a very specific stage of RNA life-cycle – the pioneer round of translation. As mentioned in the introduction, this round is mediated by CBC attached to 5′-end of mRNA in the nucleus. Obviously, plant virus RNAs, being synthesized directly in the cytoplasm, do not bind CBC. Therefore, these RNAs probably enter steady-state translation bypassing the pioneer round and consequently escaping NMD.

This hypothesis was supported by brief analysis of viral taxonomy. We have scanned the ICTV virus taxonomy database and counted number of families (and separately – genera) of DNA and RNA viruses that infect animals or plants. It turned out that there is almost the same number of DNA and RNA animal viruses (8 to 11 families and 42 to 50 genera, correspondingly). At the same time, DNA viruses are significantly less abundant among plant viruses – only 3 families compared to 9 of RNA viruses and only 13 genera compared to 55. This suggests that plant DNA-viruses cannot avoid NMD of their polycistronic RNAs, and this creates a serious handicap for plant DNA-viruses compared to plant RNA-viruses from the evolutionary point of view.

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REFERENCES

1. Culbertson, M. R., and Leeds, P. F. (2003) *Curr. Opin. Genet. Dev.*, **13**, 207-214.
2. Frischmeyer, P. A., and Dietz, H. C. (1999) *Hum. Mol. Genet.*, **8**, 1893-1900.
3. Holbrook, J. A., Neu-Yilik, G., Hentze, M. W., and Kulozik, A. E. (2004) *Nat. Genet.*, **36**, 801-808.
4. Belgrader, P., Cheng, J., Zhou, X., Stephenson, L. S., and Maquat, L. E. (1994) *Mol. Cell Biol.*, **14**, 8219-8228.
5. Cheng, J., and Maquat, L. E. (1993) *Mol. Cell Biol.*, **13**, 1892-1902.
6. Lejeune, F., Ranganathan, A. C., and Maquat, L. E. (2004) *Nat. Struct. Mol. Biol.*, **11**, 992-1000.
7. Maquat, L. E. (2004) *Nat. Rev. Mol. Cell Biol.*, **5**, 89-99.
8. Maquat, L. E. (2004) *Curr. Genom.*, **5**, 175-190.
9. Maquat, L. E. (2005) *J. Cell Sci.*, **118**, 1773-1776.
10. Gonzalez, C. I., Bhattacharya, A., Wang, W., and Peltz, S. W. (2001) *Gene*, **274**, 15-25.
11. Jofuku, K. D., and Goldberg, R. B. (1989) *Plant Cell*, **1**, 1079-1093.
12. Voelker, T. A., Moreno, J., and Chrispeels, M. J. (1990) *Plant Cell*, **2**, 255-261.
13. Van Hoof, A., and Green, P. J. (1996) *Plant J.*, **10**, 415-424.
14. Dickey, L. F., Nguyen, T. T., Allen, G. C., and Thompson, W. F. (1994) *Plant Cell*, **6**, 1171-1176.
15. Petracek, M. E., Nuygen, T., Thompson, W. F., and Dickey, L. F. (2000) *Plant J.*, **21**, 563-569.
16. Isshiki, M., Yamamoto, Y., Satoh, H., and Shimamoto, K. (2001) *Plant Physiol.*, **125**, 1388-1395.
17. Hori, K., and Watanabe, Y. (2005) *Plant J.*, **43**, 530-540.
18. Yoine, M., Ohto, M. A., Onai, K., Mita, S., and Nakamura, K. (2006) *Plant J.*, **47**, 49-62.
19. Chiu, W., Niwa, Y., Zeng, W., Hirano, T., Kobayashi, H., and Sheen, J. (1996) *Curr. Biol.*, **6**, 325-330.

20. Dorokhov, Y. L., Skulachev, M. V., Ivanov, P. A., Zvereva, S. D., Tjulkina, L. G., Merits, A., Gleba, Y. Y., Hohn, T., and Atabekov, J. G. (2002) *Proc. Natl. Acad. Sci. USA*, **99**, 5301-5306.
21. Komarova, T. V., Skulachev, M. V., Zvereva, A. S., Schwartz, A. M., Dorokhov, Yu. L., and Atabekov, J. G. (2006) *Biochemistry (Moscow)*, **71**, 846-850.
22. Napoli, C., Lemieux, C., and Jorgensen, R. (1990) *Plant Cell*, **2**, 279-289.
23. Hoshino, A., Morita, Y., Choi, J. D., Saito, N., Toki, K., Tanaka, Y., and Iida, S. (2003) *Plant Cell Physiol.*, **44**, 990-1001.
24. Amrani, N., Ganesan, R., Kervestin, S., Mangus, D. A., Ghosh, S., and Jacobson, A. (2004) *Nature*, **432**, 112-118.
25. Buhler, M., Steiner, S., Mohn, F., Paillusson, A., and Muhlemann, O. (2006) *Nature Struct. Mol. Biol.*, **13**, 462-464.
26. Skulachev, M. V., Ivanov, P. A., Karpova, O. V., Korpela, T., Rodionova, N. P., Dorokhov, Y. L., and Atabekov, J. G. (1999) *Virology*, **263**, 139-154.
27. Ivanov, P. A., Karpova, O. V., Skulachev, M. V., Tomashevskaya, O. L., Rodionova, N. P., Dorokhov, Yu. L., and Atabekov, J. G. (1997) *Virology*, **232**, 32-43.
28. Zvereva, S. D., Ivanov, P. A., Skulachev, M. V., Klyushin, A. G., Dorokhov, Yu. L., and Atabekov, J. G. (2004) *J. Gen. Virol.*, **85**, 1739-1744.
29. Dorokhov, Yu. L., Ivanov, P. A., Komarova, T. V., Skulachev, M. V., and Atabekov, J. G. (2006) *J. Gen. Virol.*, **87**, 2693-2697.