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Expression of the eRF1 translation termination factor is controlled by an autoregulatory circuit involving readthrough and nonsense-mediated decay in plants

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ABSTRACT

When a ribosome reaches a stop codon, the eukaryotic Release Factor 1 (eRF1) binds to the A site of the ribosome and terminates translation. In yeasts and plants, both over- and underexpression of eRF1 lead to altered phenotype indicating that eRF1 expression should be strictly controlled. However, regulation of eRF1 level is still poorly understood. Here we show that expression of plant eRF1 is controlled by a complex negative autoregulatory circuit, which is based on the unique features of the 3′untranslated region (3′UTR) of the eRF1-1 transcript. The stop codon of the eRF1-1 mRNA is in a translational readthrough promoting context, while its 3′UTR induces nonsense-mediated decay (NMD), a translation termination coupled mRNA degradation mechanism. We demonstrate that readthrough partially protects the eRF1-1 mRNA from its 3′UTR induced NMD, and that elevated eRF1 levels inhibit readthrough and stimulate NMD. Thus, high eRF1 level leads to reduced eRF1-1 expression, as weakened readthrough fails to protect the eRF1-1 mRNA from the more intense NMD. This eRF1 autoregulatory circuit might serve to finely balance general translation termination efficiency.

INTRODUCTION

Translation is terminated when a ribosome reaches a stop codon, and the eukaryotic Release Factor 1 (eRF1)–Release Factor 3 (eRF3) complex binds to the A site of the ribosome. eRF1 stimulates both peptide release and ribosome disassembling. Infrequently, if a transcript contains a specific signal and/or cellular conditions do not favor normal translational termination, alternative events such as readthrough, frameshift or nonsense-mediated decay (NMD) can occur at the stop codon (1).

eRF1 expression has to be strictly controlled. Low eRF1 concentration reduces termination efficiency, thereby increasing the frequency of both frameshift and readthrough, while eRF1 overexpression might be harmful by reducing readthrough frequency below a critical level and/or by causing termination at coding codons (2,3). Indeed, it has been shown that in yeast slightly reduced eRF1 protein level leads to significantly decreased protein synthesis and growth rates, and strong selection acts to maintain optimal termination efficiency in different yeast strains (4,5). Strict control of eRF1 expression is also important in plants. In *Arabidopsis thaliana*, reduced eRF1 level results in a developmental phenotype, while overexpression leads to ABA and sugar hypersensitivity (6–8). Although it has been known for decades that in prokaryotes, expression of the key termination factor RF2 is stabilized by an autoregulatory circuit (9), knowledge about eRF1 regulation in eukaryotes is still limited.

Readthrough occurs if the stop codon is bound by a near cognate tRNA, and translation elongation is continued till the next in-frame stop codon (referred to as 'next stop'). Readthrough frequency is very low at normal stop codons but it can be relatively high if the sequence context of the stop codon supports readthrough (readthrough stop context). The six nucleotides downstream of the stop codon are critical for readthrough. Mutational studies revealed that the consensus readthrough signal is the stop-CARYYA sequence, where the +4C, the first nucleotide downstream of the stop codon, is the most important readthrough inducing element (10). Sequences upstream of the stop codon and secondary structures in the 3′ untranslated region (3′UTR) can also stimulate readthrough (11–13). Readthrough is essential for the gene expression of several viruses (14). In ad-

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dition, it plays an important role in eukaryotic gene regulation by producing C-terminally extended protein forms, which can act antagonistically to the normally terminated protein forms or localize in different compartment (14–18). Moreover, at least in fungi, readthrough is involved in adaptation and evolvability (19).

NMD is another alternative event that can occur if translation termination is inefficient. It is a translation termination coupled conserved eukaryotic quality control system that plays a critical role in the identification and degradation of premature termination codon containing aberrant mRNAs and controls the expression of several normal transcripts. NMD is induced when the eRF1–eRF3 complex has been recruited to the stop codon, but termination is still slow because the termination stimulating 3′UTR signals are absent and/or termination inhibitory signals are present in the 3′UTR (20,21). NMD triggers rapid degradation of the mRNA without inhibiting the release of the synthesized peptide. Unusually long 3′UTRs and introns >50 nt downstream of the stop codon are the most important NMD-inducing signals (NMD *cis* elements). Long 3′UTR induces NMD because it inhibits the termination stimulating binding of the poly (A) binding protein (PABP) to the eRF3 component of the termination complex. In this case eRF3 interacts with the up-frameshift 1 (UPF1) NMD factor, which then recruits UPF2 and UPF3 to the terminating ribosome. The UPF1-2-3 NMD complex triggers the rapid degradation of the mRNA. Long 3′UTR induces NMD in all eukaryotes (22–24), whereas 3′UTR located introns trigger NMD efficiently only in vertebrates, plants and certain filamentous fungi (25–27). In certain eukaryotes including vertebrates and plants, splicing leads to the deposition of a protein complex called exon–junction complex (EJC) 20–24 nt upstream of the new exon–exon boundary (28,29). The EJC forms a binding platform for the UPF3 and UPF2 NMD factors. During the first round of translation the ribosome removes the EJCs located in the 5′UTR, the coding region and the first 20–25 nt of the 3′UTR, but fails to displace EJCs bound more downstream in the 3′UTR. These 3′UTR-bound EJCs stimulate vertebrate and plant NMD, presumably because they increase the local concentration of UPF3 and UPF2, thereby facilitating the formation of the NMD complex. In addition to eliminating premature termination codon containing aberrant transcripts, NMD also reduces the stability of normal mRNAs having NMD inducing *cis* elements in their 3′UTRs.

Translation termination, readthrough and NMD are interconnected. eRF1 and near cognate tRNAs compete for the stop codon, hence reduced termination efficiency leads to enhanced readthrough (13). Moreover, in yeast and mammals, readthrough can protect mRNAs that contain NMD *cis* elements in their 3′UTR from NMD-mediated rapid degradation if the stop codon of the ORF is in a readthrough context and the next stop is not in an NMD-inducing position (30,31). This specific 3′UTR composition, when (i) the 3′UTR contains NMD inducing *cis* elements, (ii) the stop codon is in readthrough context and (iii) the next stop codon is not in an NMD-inducing position, will be referred to as RT-NMD 3′UTR structure. In this situation, the readthrough can protect the mRNA from NMD. It is postulated that when readthrough occurs at the stop codon, the translating ribosome removes NMD stimulatory signals including EJC from the 3′UTR region till the next stop codon, thereby rescuing the mRNA from NMD (30).

All three events: normal termination, readthrough and NMD are physiologically important. Proper translation termination is essential in all eukaryotes and a certain level of readthrough is also required for viability in yeast and *Drosophila* (15,17,32). NMD-deficiency is lethal in *Drosophila*, vertebrates and plants (33–35). In plants, NMD plays a critical role in the control of pathogen response and the regulation of alternative splicing (36–41). Plant NMD also plays a role in the regulation of RNA silencing pathway (42).

Translation termination has been barely studied in plants. Interestingly, eRF1 is a single copy gene in most model eukaryotes, but it is present in three copies (*eRF1-1*, *eRF1-2* and *eRF1-3*) in the model plant *A. thaliana* (43). Although the three copies share high similarity, and each can complement eRF1 deficiency in yeast, they are not completely redundant (8,43). These observations suggest that eRF1 regulation might be especially complex in plants.

It is not known whether in plants, like in yeast and mammals, readthrough can protect transcripts from NMD. To better understand the connections between normal termination, readthrough and NMD in plants, we searched for plant transcripts that have a RT-NMD 3′UTR structure.

Here we show that in *Arabidopsis* eRF1-1 mRNA is the only transcript having a RT-NMD 3′UTR structure, and that this feature is conserved among all seed plants. Functional analyses of the eRF1-1 RT-NMD 3′UTR revealed that in plants overall eRF1 protein level is stabilized by a complex autoregulatory circuit, in which both readthrough and NMD play a critical role. The mechanism, the function and the evolution of this sophisticated eRF1 autoregulatory system will be discussed.

MATERIALS AND METHODS

The list of primers, the schematic representations of the constructs with the explanation of their names, plant lines and the detailed descriptions of the methods are available at Supplementary Materials and Methods.

Agroinfiltration transient gene expression assays

Agroinfiltration of *Nicotiana benthamiana* leaves is the most efficient transient gene (co-) expression system for plants (44). Briefly, to co-express different genes, each of them is cloned into Bin61S (45) binary plasmid (or into the derivative of Bin61S) and then each vector is introduced into an *Agrobacterium tumefaciens*. The *Agrobacterium* cultures, which are grown independently, are mixed, and the mixture is injected (agroinfiltrated) into the leaves of *N. benthamiana* plants. *N. benthamiana* leaves were agroinfiltrated with a mixture of bacterium cultures (OD_{600} of each culture was 0.4, or in the case of P14 0.2). Agroinfiltration induces strong RNA silencing (RNAi) response, which could reduce the expression dramatically. To prevent activation of silencing the P14 viral silencing suppressor is co-expressed (46). In our assays, P14 has a dual role, it inhibits silencing and it is used as an internal control for western and

northern assays. We have shown that P14 does not modify NMD (25). Thus as a minimum, two bacteria are mixed for agroinfiltration assay, one expressed the P14 suppressor, whereas the second bacterium expressed the test (or the control) construct. GFP (green fluorescent protein) fluorescence was detected by using a handhold long-wave ultraviolet lamp (UV products, Upland, B-100 AP). Under UV illumination the uninfiltrated patches show red and the GFP infiltrated patches show green fluorescence. For photos, FL-D D-60 fluorescent filter was used (Hoya).

U1DN assays

For UPF1 dominant-negative assay, at least three bacteria are mixed, one expresses the P14, the second one the test gene and the third one expresses a dominant-negative version of *Arabidopsis* UPF1 (U1DN). U1DN inhibits NMD, thus the NMD sensitive test transcripts are overexpressed in U1DN co-infiltrated samples (25).

VIGS-agroinfiltration NMD assays

Virus-induced gene silencing (VIGS) was used to transiently silence UPF1 (47). *N. benthamiana* plants were infected with *Tobacco Rattle Virus* VIGS vector containing a segment from the UPF1 or from the PDS (phytoene desaturase) gene (called UPF1 and control silenced plants, respectively).

Plant transformation

Agrobacterium-mediated transformation was carried out to generate transgenic plants. Floral dip and leaf disc transformation methods were used to generate transgenic *Arabidopsis* and *N. benthamiana* lines, respectively.

Western and RNA gel blot assays

Monoclonal antibodies (anti-HA and anti-Myc peroxidase, Roche and Sigma, respectively) were used for chemiluminescence protein detections according to the manufacturer's instructions (ECL, Promega). To quantify protein expression western blots were scanned with ChemiDoc MP and analyzed with ImageLab software (Bio-Rad).

RNA gel blot assays and quantifications were described (25). GFP and P14 polymerase chain reaction (PCR) fragments labeled with random priming method were used as probes for northern analyses. Phosphorimage measurements were used to quantify mRNA expression.

RT-PCR assays

For quantitative RT-PCR studies cDNAs were reverse transcribed using RevertAid First Strand cDNA Synthesis kit (Thermo Scientific). qPCR was carried out with Fast Start Essential DNA Green Master (Roche) in a Light Cycler 96 (Roche) Real-Time PCR machine.

Readthrough assays

To estimate readthrough frequency, NAN-GUS readthrough assays were conducted as described (48). Readthrough reporter constructs, in which the NAN and GUS reporter genes were separated with different stop contexts, were co-infiltrated with P14 or with P14 and another construct that expressed eRF1 protein. The NAN and GUS activities were measured at 3 days post inoculation (d.p.i).

RESULTS

eRF1-1 mRNA has a RT-NMD 3′UTR

Plant NMD targets transcripts with long 3′UTR (>350–400 nt) or with an intron more than 50 nt downstream of the stop codon (22,25,49). Although ∼20% of *Arabidopsis* transcripts are potential NMD targets (50), only few % of the genes are overexpressed in NMD-deficient mutants (36,51), suggesting that several transcripts evade NMD (however NMD also downregulates the expression of several alternative splicing isoforms and long-noncoding mRNAs (40,51,52)). To test if readthrough plays a role in NMD evasion in plants, the *Arabidopsis* genome was searched for transcripts having a readthrough stop context (where the stop codon is followed by the CARYYA consensus sequence) and a 3′UTR with an NMD *cis* element (3′UTR > 375 nt and/or an intron >50 nt downstream of the stop codon). We found only one *Arabidopsis* gene (AT5G47880) that expresses an mRNA with a readthrough stop context and an NMD sensitive 3′UTR (Figure 1A and Supplementary Table S1). Thus, CARYYA readthrough element mediated NMD evasion is uncommon in plants.

Interestingly, AT5G47880 encodes the eRF1-1 termination factor. It has been reported that eRF1-1 mRNA has a stop context that is highly similar to the *Tobacco mosaic virus* readthrough context (TMV: *TAG*CAATTA, eRF1-1: *TGA*CAATCA) (43). Moreover, we found that eRF1-1 mRNA has a 378 nt long 3′UTR that contains a potentially NMD-inducing intron 130 nt downstream from the stop codon (Figure 1A). In mammals, readthrough at the main stop codon can protect an mRNA from 3′UTR induced NMD when the next stop is not in an NMD-inducing position (30). Relevantly, the next stop of eRF1-1 mRNA is near the polyadenylation site and very close to the 3′UTR intron (<25 nt upstream) (Figure 1A), suggesting that it does not induce NMD.

Although the coding region of the three *Arabidopsis* eRF1 transcripts is highly conserved, the structure and the sequence of their 3′UTRs is markedly different (Figure 1A). The eRF1-1 transcript has a potential RT-NMD 3′UTR, while eRF1-2 and eRF1-3 have short and intronless 3′UTRs. Evolutionary conservation of this unique 3′UTR structure of eRF1-1 might suggest that it is physiologically important. Indeed, we found that the eRF1 gene is present in at least two copies in all monocots and dicots. Each species contains two types of eRF1 genes (Figure 1A; Supplementary Table S2 and Supplementary Figure S1A and B), one that has a 3′UTR structure similar to the *Arabidopsis* eRF1-1 (these genes will be referred to as eRF1-1-like genes) and a second that has a 3′UTR similar to *Arabidopsis* eRF1-2 and eRF1-3 (called eRF1-2-like genes). Some species have only one eRF1-1-like and one eRF1-2-like gene, while others contain more than one copy of the

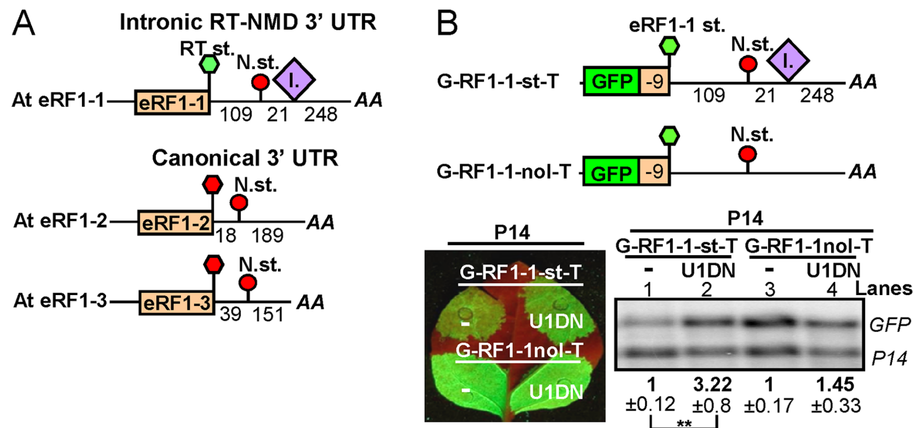

Figure 1. Plants express two types of eRF1 transcripts and the 3′UTR of eRF1-1 induces NMD. (**A**) Schematic, non-proportional representation of the *Arabidopsis* eRF1 mRNAs. Green and red hexagons show stop codons in readthrough (RT st.) and non-readthrough context, respectively. The next stop is shown as a red circle. The 3′UTR intron is shown as a purple diamond. Numbers indicate the distance in nucleotides. (**B**) The 3′UTR of *Arabidopsis* eRF1-1 induces intron-based NMD. The stop context and terminator of the *Arabidopsis* eRF1-1 and its intronless version were fused to a GFP reporter gene (G-RF1-1-st-T and G-RF1-1noI-T), and then these constructs were co-expressed only with the P14 internal control (−) or with P14 and a dominant-negative mutant of UPF1 (U1DN) in *Nicotiana benthamiana* leaves. The reporters are shown as transcripts. Photo and RNA samples were taken at 3 days post infiltration (d.p.i). Photo was taken under UV light, thus the non-infiltrated parts of the leaf are red due to the autofluorescence of chlorophyll, while the GPF expressing agroinfiltrated patches show green fluorescence. RNA gel blot was hybridized with GFP and P14 probes (italics). To quantify RNA samples, at each lane the signal of the reporter mRNA (GFP probe) was normalized to the corresponding P14 signal (GFP/P14 signal). Mean values were calculated from three independent samples ($n = 3$). To measure the NMD sensitivity of the test mRNAs, the GFP/P14 ratio of the G-RF1-1-st-T+P14 sample (lane 1) was taken as 1 and the GFP/P14 ratio of the corresponding U1DN co-infiltrated sample (lane 2) is shown relative to it. Similarly, the GFP/P14 ratio of the G-RF1-1noI-T +P14 sample (lane 3) was taken as 1 and the GFP/P14 ratio of the corresponding U1DN co-infiltrated sample (lane 4) is shown relative to it (± shows standard deviation, SD). Paired *t*-tests were used to calculate the significance of the differences ($P < 0.05$, $P < 0.01$ and $P < 0.001$ significance levels are shown as *, ** and ***). Note that expression of NMD target transcripts leads to weak fluorescence and low GFP/P14 signal in the absence of U1DN (−), while U1DN co-expression results in enhanced green fluorescence and increased GFP/P14 signal.

eRF1-1-like or the eRF1-2-like gene (or both) (Supplementary Table S2).

All eRF1-1-like transcripts contain a readthrough stop context, a 3′UTR with a potentially NMD-inducing intron and a next-stop in a non-NMD inducing position, while the eRF1-2-like mRNAs have a normal stop context and an intronless 3′UTR (referred to as intronic RT-NMD and canonical 3′UTR structure, respectively). These data suggest that in higher plants strong selection acts to retain both types of eRF1 transcripts and prevent expression of eRF1 transcripts that differ from these two types (Figure 1A and Supplementary Table S2).

Further characteristic differences can be also identified between eRF1-1- and eRF1-2-like mRNAs. The readthrough stop context of eRF1-1-like transcripts is extremely conserved, the 6 nt downstream from the stop (CAATCA) is identical in (almost) all eRF1-1 mRNAs. By contrast, these positions are not conserved in eRF1-2-like transcripts. eRF1-1-like transcripts also contain additional readthrough promoting features like a TAG stop codon and A nucleotides at the −2 and −1 positions. Moreover, the eRF1-1-like mRNAs have longer 3′UTRs, and the distance between the stop and the next stop codons is bigger in these transcripts (Supplementary Figure S1A).

The fact that eRF1-1 is the only gene in *Arabidopsis* with a RT-NMD 3′UTR, and that all monocots and dicots express eRF1-1 mRNAs with an intronic RT-NMD 3′UTR indicate that this unique structure plays an important, conserved role in plants. To understand the function of this unique structure, we analyzed the RT-NMD 3′UTR of *Arabidopsis* eRF1-1.

The 3′UTR intron of eRF1-1 induces NMD

To test whether the intronic RT-NMD 3′UTR of eRF1-1 indeed induces NMD, we cloned the stop context and the terminator region of *Arabidopsis* eRF1-1 (from −9 nt relative to the first nt of the stop codon to +200 nt relative to the polyadenylation site) downstream of a GFP reporter that lacks its own stop codon (G-RF1-1-st-T, for GFP with the eRF1-1 stop context and terminator region) (Figure 1B) and then the NMD sensitivity of the reporter transcript was tested in a UPF1 dominant-negative (U1DN) agroinfiltration assay (25) (U1DN assay is described in 'Materials and Methods' section). The G-RF1-1-st-T construct was co-expressed in *N. benthamiana* leaves with the P14 silencing suppressor, which was used in these experiments as an internal control or with P14 and U1DN, a dominant-negative mutant of UPF1 that inactivates NMD (P14 is co-expressed in each experiment and it is shown on the figures, but it will not be mentioned in the main text. The role of P14 in these assays is described in 'Materials and Methods' section). The G-RF1-1-st-T mRNA level was significantly higher and the green fluorescence was more intense in the U1DN co-expressed sample (Figure 1B, compare lanes 1–2 and the left and the right side of the upper part of the leaf) indicating that the eRF1-1 3′UTR triggers NMD. Moreover, the removal of the intron (G-RF1-1noI-T) severely decreased the NMD sensitivity of the reporter mRNA (Figure 1B). Thus we concluded that the 3′UTR located intron of eRF1-1 induces NMD. Control experiments confirmed that the eRF1-1 3′UTR intron was efficiently spliced (Supplementary Figure S1C and D).

The *Arabidopsis* eRF1-2 and eRF1-3 transcripts have canonical 3′UTRs (Figure 1A). As expected, their 3′UTRs did not induce NMD (Supplementary Figure S2A).

Readthrough partially rescues the eRF1-1 mRNAs from NMD

In mammals, readthrough can inhibit NMD (30). However, the G-RF1-1-st-T mRNA, which contains both the readthrough stop context and the NMD-inducing 3′UTR of eRF1-1, is targeted by plant NMD (Figure 1B). This result suggests that the readthrough stop context of eRF1-1 does not, or only partially protects the reporter transcript from NMD.

To distinguish between these two possibilities, a non-readthrough control (G-RF1-1C/G-T) was generated from the G-RF1-1-st-T construct, by mutating the +4C to G (Figure 2A). Relevantly, this non-readthrough reporter mRNA accumulated to a significantly lower level than G-RF1-1-st-T transcript (Figure 2B, compare lanes 1 and 3). In line, the green fluorescence was weaker in the G-RF1-1C/G-T expressing leaves. However, when NMD was inactivated, the two constructs expressed to comparable levels (Figure 2B, compare lanes 2 and 4). These data indicate that the eRF1-1 readthrough stop context partially rescues the transcript from NMD.

Next we studied the relevance of the position of the next stop. In theory, the next stop of eRF1-1 is not in an NMD-inducing position. To test this, a reporter construct was generated (G-RF1-1-nost-T), in which the readthrough stop codon of the G-RF1-1-st-T construct was changed to the CAG coding codon, thereby turning the next stop into the main stop codon of the transcript. This reporter expressed to high levels, and its expression was insensitive to NMD (Figure 2B lanes 7 and 8), indicating that NMD is not induced when translation is terminated at the next stop of eRF1-1.

To analyze how the position of the next stop affects the eRF1-1 3′UTR induced NMD, we incorporated an artificial next stop into the 3′UTR of the G-RF1-1-st-T construct into an NMD-inducing position, close to the readthrough stop (G-RF1-1-ast-T). As insertion of a next stop into an NMD-inducing position strongly reduced the mRNA level and the green fluorescence (Figure 2B, compare lanes 1 and 5), we concluded that readthrough can only rescue the transcript from NMD if the next stop is not in an NMD inducing position.

Taken together, our data suggest that the *Arabidopsis* eRF1-1 has a functional intronic RT-NMD 3′UTR as the readthrough stop context partially rescues the reporter transcripts from NMD. Moreover, similar reporter gene assays revealed that the readthrough stop contexts of *N. benthamiana* eRF1-1 transcript also efficiently rescued the reporter mRNAs from its 3′UTR induced NMD (Supplementary Figure S2B). Thus, the *N. benthamiana* eRF1-1 mRNAs also contain a functional RT-NMD 3′UTR.

Based on these results, we hypothesized that the endogenous eRF1-1 mRNA, like the G-RF1-1-st-T reporter transcript, is a moderate target of NMD, as readthrough can only partially protect the eRF1-1 transcript from NMD. Indeed, quantitative RT-PCR assays showed that eRF1-1 was 2–7-fold overexpressed in different NMD deficient *Arabidopsis* lines, as well as in UPF1-silenced *N. benthamiana* plants (Figure 2C and D). We also analyzed the expression of eRF1-1 mRNA in the available transcriptome wide studies conducted in various NMD-deficient *Arabidopsis* lines (36,37,51–53). The eRF1-1 mRNA was (one of the only four transcripts that was) overexpressed in each experiment, while the eRF1-2 or the eRF1-3 was not upregulated in any of these studies (Supplementary Table S3). These data confirm that the eRF1-1 (but not the eRF1-2 and eRF1-3) mRNA is downregulated by NMD.

The efficiency of NMD protection depends on readthrough frequency

The stop context of the angiosperm eRF1-1 genes is extremely conserved (Supplementary Figure S1A and Supplementary Table S2) suggesting that strong selection acts to keep the optimal readthrough frequency. To study the readthrough frequency of eRF1-1, NAN-GUS readthrough assays were carried out to compare the readthrough level of the eRF1-1 stop context, the +4 C/G non-readthrough eRF1-1 stop context and the well-characterized TMV stop contexts (48). As Figure 3A shows, the TMV stop context (stop context: from −9 to +12 nt) triggers significantly higher levels of readthrough than the eRF1-1 stop context, while the readthrough of the eRF1-1 stop context was higher than the readthrough of the +4 C/G non-readthrough stop context. Thus, the eRF1-1 stop context allows moderate level of readthrough. This finding is consistent with the previous result that incorporation of the eRF1-1 stop context downstream of a reporter transcript leads to a low but detectable level of readthrough in rabbit reticulocyte *in vitro* translation system (43).

To test how readthrough frequency affects NMD inhibition, the eRF1-1 readthrough stop context of the G-RF1-1-st-T construct was replaced by the TMV readthrough context (G-TMVst1-1-T). We found that the TMV readthrough stop context, unlike the eRF1-1 context, fully protected the reporter transcripts from eRF1-1 3′UTR induced NMD (Figure 3B and Supplementary Figure S3).

Thus, the extent of NMD rescue depends on the intensity of readthrough. The highly conserved stop context of eRF1-1 allows moderate readthrough frequency that can only partially protect the mRNAs from NMD.

Overexpression of eRF1 inhibits readthrough in plants

Our data suggest that both readthrough and NMD affect eRF1-1 mRNA, and consequently eRF1 protein levels. Thus we wanted to study how in turn eRF1 protein concentration affects the frequency of readthrough and the intensity of NMD. To test the effect of eRF1 protein overexpression on readthrough frequency, a readthrough reporter construct was co-expressed with *N. benthamiana* eRF1-1 and *Arabidopsis* eRF1-1 (NbRF1-1 and AtRF1-1) in *N. benthamiana* leaves. Relevantly, both eRF1 proteins reduced readthrough frequency severely (Figure 4A).

In *N. benthamiana* at least three eRF1 paralogs exist. NbeRF1-1 mRNA has an RT-NMD 3′UTR structure, while NbeRF1-2 and NbeRF1-3 transcripts have canonical

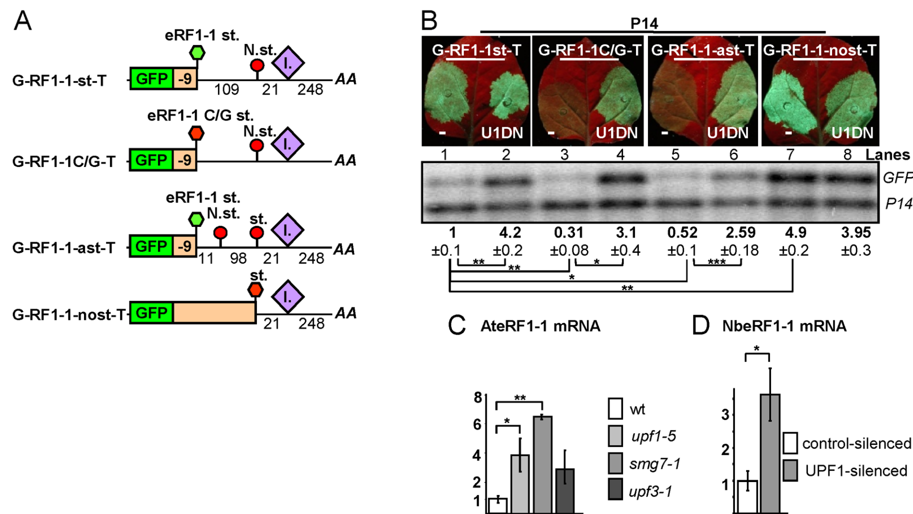

Figure 2. The readthrough of eRF1-1 stop context partially rescues the transcript from eRF1-1 3′UTR induced NMD. (**A**) Schematic, non-proportional representation of the used reporter transcripts. Readthrough and non-readthrough stop codons are shown as green and red hexagons. Red circle indicates the next stop and purple diamond the 3′UTR intron. The stop context and the terminator of the *Arabidopsis* eRF1-1 was cloned downstream of a GFP (G-RF1-1-st-T). As controls, a non-readthrough (G-RF1-1C/G-T) and an artificial next stop containing (G-RF1-1-ast-T) derivatives were created from the G-RF1-1-st-T construct. To test the efficiency of termination at the next stop, the stop was replaced with a CAG codon (G-RF1-1nost-T). (**B**) The eRF1-1 readthrough stop context partially rescues the reporter transcripts from eRF1-1 3′UTR induced NMD. Samples were taken and analyzed as described at Figure 1B ($n = 3$, ± shows SD). The GFP/P14 ratio of the G-RF1-1-st-T (–) sample was taken as 1 and the GFP/P14 ratios of other samples are shown relative to it. Paired *t*-tests were used to calculate the significance of the differences. If one sample was used in multiple comparisons, Bonferroni correction was applied. Note that the non-readthrough and the artificial next stop containing transcripts are strong targets of NMD, the G-RF1-1-st-T mRNA is a moderate target of NMD and the G-RF1-1nost-T mRNA is not regulated by NMD. (**C**) The eRF1-1 mRNA is overexpressed in NMD-deficient *Arabidopsis* mutants. eRF1-1 mRNA level was measured by qRT-PCR in wild-type (wt) and in weak (*upf1-5* and *upf3-1*) and strong (*smg7-1*) NMD mutant *Arabidopsis* lines. Three samples were analyzed from each lines ($n = 3$), and the expression of the mutants are shown relative to the wt line. Bonferroni correction was applied. (**D**) The eRF1-1 mRNA is overexpressed in NMD-deficient *Nicotiana benthamiana* plants. The eRF1-1 mRNA level was measured by qRT-PCR in control-silenced and UPF1-silenced plants.

3′ UTRs (Supplementary Table S2). To test if all three eRF1 proteins can terminate translation, the activity of these proteins was studied in readthrough assays. All three *N. benthamiana* eRF1 proteins reduced readthrough to a similar extent indicating that these proteins play a similar role in termination (Supplementary Figure S4B). The different eRF1 proteins expressed to comparable levels (Supplementary Figure S4A).

Taken together, our data suggest that in *N. benthamiana* (and presumably in many other plant species), the frequency of readthrough negatively correlates with the total eRF1 protein level. Overexpression of eRF1 is also sufficient to reduce readthrough frequency in certain mammalian cell lines, while in yeasts, overexpression of both eRF1 and eRF3 termination factors is required to reduce readthrough frequency (13,54). Thus we propose that in plants (and in certain mammalian cells), the concentration of the eRF1 protein limits the efficiency of translational termination.

The C-terminally extended version of eRF1-1 protein might not be involved in translation termination

If readthrough occurs at the stop codon of the NbeRF1-1 mRNA, a C-terminally extended protein is translated (referred to as NbRF1-1ext. for extended). To clarify whether this putative readthrough product is functional in translation termination, an artificial NbRF1-1ext. was generated by replacing the stop codon with a CAG codon. Although NbRF1-1ext. protein accumulates to high level (Supplementary Figure S4A), its co-expression did not decrease readthrough frequency (Supplementary Figure S4C), suggesting that the C-terminally extended version of the eRF1-1 protein does not act as a translation termination factor. In line, the NbRF1-1ext. protein was also inactive in other translation termination assays (see below, Supplementary Figure S5C).

eRF1 overexpression intensifies NMD

To test how eRF1 level affects NMD, NbRF1-1 and NbRF1-3 proteins were co-expressed with two different GFP-based NMD reporter constructs having normal, non-readthrough stop context. Surprisingly, overexpression of both eRF1 proteins led to significantly reduced accumulation of the NMD reporter transcripts (Figure 4B and C). Moreover, overexpression of NbeRF1-2 also led to decreased NMD reporter mRNA levels (Supplementary Figure S5A). By contrast, co-expression of the eRF1 proteins did not result in significantly reduced expression of the non-NMD target GFP mRNA (Figure 4D). Finally, NbRF1-3 selectively reduced the expression of the GFP-based NMD reporter mRNA when NbRF1-3 was co-expressed with both the GFP control and the NMD sensor test constructs (Figure 4E and Supplementary Figure S5B). These results suggest that enhanced eRF1 protein level leads to more intense NMD and consequently to reduced NMD reporter transcript levels. Indeed, we found that NbeRF1-3 co-expression failed to reduce the accumulation of NMD re-

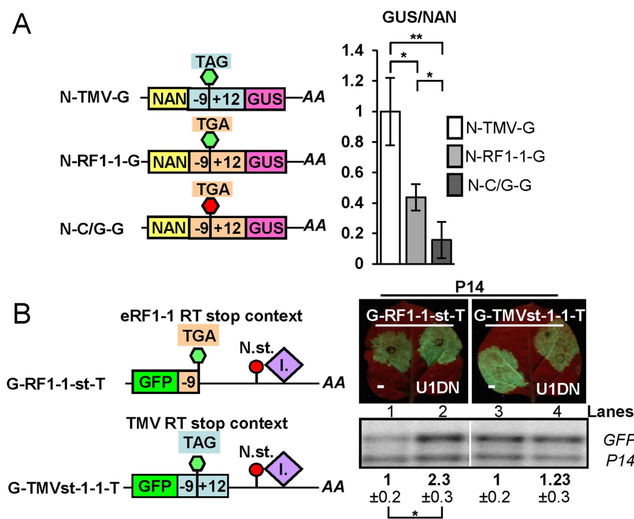

Figure 3. Efficient readthrough fully rescues the reporter transcripts from eRF1-1 3′UTR induced NMD. (**A**) The eRF1-1 stop context allows moderate level of readthrough. The readthrough frequency of the TMV readthrough context (from −9 nt to +12 nt if the first nt of the stop codon is +1), the eRF1-1 readthrough context and its non-readthrough version (+4 C mutated to G) were measured in readthrough assays ($n = 3$, the error bar shows SD, paired *t*-tests with Bonferroni corrections were used to calculate the significance). The stop contexts were cloned between the NAN and GUS reporters (N-TMV-G, N-RF1-1-G and N-C/G-G) and the constructs were infiltrated into *Nicotiana benthamiana* leaves. The ratios of GUS/NAN activities were compared. The TMV and the eRF1-1 stop contexts are in light blue and apricot, respectively. (**B**) The TMV readthrough stop, unlike the eRF1-1 stop, fully protects the transcripts from eRF1-1 3′UTR induced NMD. The eRF1-1 stop context (from −9 to +12 nt) of the G-RF1-1-st-T reporter was replaced with the TMV context (G-TMVst-1-1-T). U1DN co-expression assays were conducted and analyzed as described at Figure 1B.

porter mRNA when NMD was inactivated by co-expressing U1DN (Figure 4F). We speculate that high eRF1 level intensifies NMD by an unknown but specific mechanism. Alternatively, enhanced eRF1 protein expression leads to more intense translation in general. As NMD is a translation coupled quality control system, accelerated translation could lead to more efficient NMD-mediated degradation of the NMD target transcripts (55).

In summary, in plants, both readthrough frequency and NMD intensity depend on the concentration of eRF1 protein: enhanced eRF1 level leads to reduced readthrough and more intense NMD.

Model of negative autoregulation of eRF1

Based on the findings that (i) readthrough can partially protect transcripts from the eRF1-1 3′UTR induced NMD, and that (ii) high eRF1 protein levels inhibit readthrough and stimulate NMD, we hypothesized that in plants, the special 3′UTR structure of eRF1-1 allows negative autoregulation of eRF1. We assume that enhanced eRF1 protein levels reduce the frequency of the NMD protecting readthrough of the eRF1-1 mRNA and intensify NMD. Consequently, NMD degrades eRF1-1 mRNA more efficiently, leading to decreased eRF1-1 transcript level and reduced eRF1-1 protein synthesis rate, thereby restoring normal overall eRF1 protein level (Figure 5A, also see 'Discussion' section).

The key predictions of this eRF1 autoregulatory model are that (i) enhanced eRF1 protein level reduces eRF1-1 expression, (ii) reduced eRF1 protein level results in increased eRF1-1 expression, (iii) eRF1 overexpression reduces the eRF1-1 level in an NMD-dependent manner and that (iv) the eRF1 protein targets the RT-NMD 3′UTR of eRF1-1. Various transient and transgenic experiments were conducted to test these predictions.

Altered eRF1 protein expression leads to compensatory changes in eRF1-1 mRNA expressions

To test if enhanced eRF1 protein level decreases the expression of eRF1-1 mRNA, expressions of eRF1-1 and eRF1-2 transcripts were measured in *N. benthamiana* leaves in which NbRF1-3 or AtRF1-1 proteins were overexpressed. Overexpression of both eRF1 proteins led to reduced NbeRF1-1 mRNA level but did not modify the expression of the canonical 3′UTR containing NbeRF1-2 mRNA (Figure 5B, left panel and Supplementary Figure S6A).

The effect of reduced eRF1 protein level was studied in the previously described *eRF1-2 Arabidopsis* null mutant (7). The autoregulatory model predicts that the eRF1-1 mRNA is overexpressed in the *eRF1-2* mutant to compensate for the absence of eRF1-2 protein. Indeed, in the *eRF1-2* mutant the eRF1-1 mRNA is upregulated, while the canonical 3′UTR containing eRF1-3 transcripts accumulate to normal levels (Figure 5C).

eRF1 protein overexpression reduces eRF1-1 mRNA expression in an NMD-dependent manner

To study the role of NMD in eRF1 autoregulation, NbRF1-3 was expressed in *N. benthamiana* leaves in the presence and absence of U1DN. NbRF1-3 overexpression significantly reduced the eRF1-1 mRNA levels when NMD was active (Figure 5B right panel). By contrast, eRF1-1 mRNA was upregulated when NbRF1-3 and U1DN were co-expressed (or when only U1DN was expressed). Thus, eRF1 protein reduces eRF1-1 mRNA expression in an NMD-dependent manner. Note that neither the NbRF1-3 expression nor the NbRF1-3 and U1DN co-expression altered the level of the canonical 3′UTR containing NbeRF1-2 mRNA (Supplementary Figure S6B).

The eRF1 protein acts on the RT-NMD 3′UTR of eRF1 mRNA

To test if eRF1 protein regulates eRF1-1 transcript levels by acting on the RT-NMD 3′UTR, the G-RF1-1-st-T reporter transcript, in which the GFP reporter is fused to the RT-NMD 3′UTR of *Arabidopsis* eRF1-1, was co-expressed with the *N. benthamiana* eRF1 proteins. All three eRF1 proteins (but not NbeRF1-1ext. protein) led to severely reduced reporter mRNA levels (Supplementary Figure S5C). Moreover, NMD was required for the reduction. As Figure 5D shows, NbRF1-3 co-expression led to dramatically reduced G-RF1-1-st-T mRNA levels when NMD was active, while it did not reduce the expression of reporter mRNA when U1DN was co-expressed (compare lane 1 to

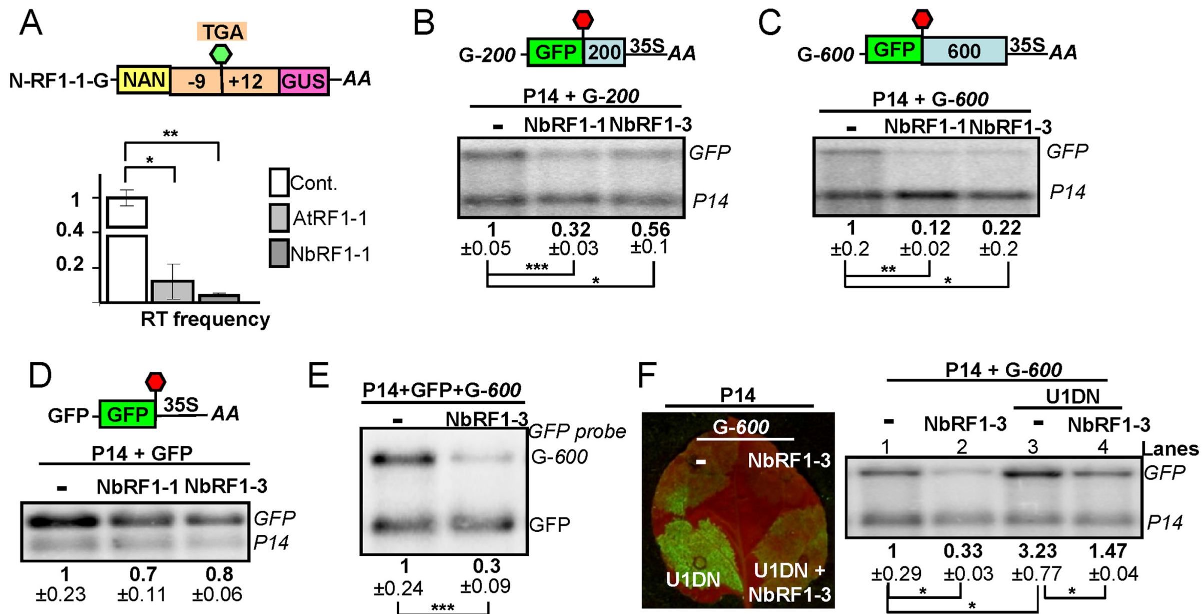

Figure 4. Overexpression of eRF1 proteins leads to reduced readthrough and more intense NMD. (**A**) eRF1 proteins inhibits readthrough. HA-tagged versions of the *Arabidopsis* eRF1-1, the *Nicotiana benthamiana* eRF1-1 and eRF1-3 proteins (AtRF1-1, NbRF1-1, NbRF1-3) were co-expressed with N-RF1-1-G readthrough reporter construct, in which the stop context (from −9 to +12 nt) of *Arabidopsis* eRF1-1 was cloned between the NAN and GUS. The assay was conducted as described at Figure 3A. (**B–E**) eRF1 proteins stimulate NMD. NMD reporter (G-*200*, G-*600*) or GFP control constructs (GFP) were co-expressed with P14 internal control (−) or with P14+NbRF1-1 or P14+NbRF1-3. The 3′UTR of the GFP control transcript is ∼145 nt long (it is derived from the 35S terminator), while the G-*200* and G-*600* GFP-based NMD reporter mRNAs, which contain a 200 nt or a 600 nt long stuffer sequence between the stop codon and the 35S 3′UTR sequence, have ∼345 nt and 745 nt long 3′UTRs, respectively. (E) eRF1 overexpression selectively reduces the expression of the NMD sensitive reporter mRNA. GFP control and G-*600* NMD reporter construct were co-expressed with P14 or with P14+NbRF1-3. The RNA gel blot was hybridized with only GFP probe and G-*600*/GFP transcript levels were measured ($n = 6$). Similar blot hybridized with both GFP and P14 probes is shown as Supplementary Figure S5B. (**F**) eRF1 overexpression reduces the expression of G-*600* mRNA in an NMD-dependent manner. G-*600* reporter was co-expressed with P14 (−), with P14+ a dominant-negative mutant of UPF1 (U1DN), with P14+NbRF1-3 or with P14+U1DN+NbRF1-3. Note that eRF1 overexpression fails to reduce the G-*600* level in the U1DN co-expressed sample. The assays were analyzed as described at Figure 2B.

3 and 4). Similar results were obtained with a reporter transcript that contained the RT-NMD 3′UTR of *N. benthamiana* eRF1-1 (Supplementary Figure S6C). These data indicate that eRF1 overexpression destabilizes the eRF1-1 RT-NMD 3′UTR containing reporter mRNAs in an NMD-dependent manner. Consistently, NbRF1-3 co-expression failed to reduce G-RF1-1-st-T transcript levels in UPF1-silenced (NMD-deficient) *N. benthamiana* plants (Supplementary Figure S6D, compare lanes 6 and 8).

As enhanced eRF1 protein level down-regulates in an NMD dependent manner both the endogenous eRF1-1 mRNA and the RT-NMD 3′UTR containing reporter transcript (Figure 5B and D), we conclude that the RT-NMD 3′UTR plays a key role in the autoregulation, it ‘senses’ the eRF1 protein concentration.

eRF1 protein concentration regulates the expression of eRF1-1 at both mRNA and protein level

The presented eRF1 autoregulatory circuit is hypothesized to stabilize overall eRF1 protein levels. Unfortunately, we failed to raise an eRF1-1 specific antibody, therefore we could not monitor expression of the endogenous eRF1-1 protein. Instead, transient and transgenic assays were conducted to study how eRF1 overexpression modulates protein levels of a tagged eRF1-1. The coding and terminator region of *Arabidopsis* eRF1-1 was fused to a Myc tag (Myc-RF1-1-st-T), and then it was transiently co-expressed with U1DN, NbRF1-3 or NbRF1-3 and U1DN (Figure 5E). A Myc-tagged P14 (Myc-P14) was used as an internal control. U1DN co-expression led to significantly enhanced Myc-RF1-1-st-T transcript and protein expression (Figure 5E, compare lanes 1 and 3). Moreover, we found that NbRF1-3 co-expression resulted in downregulation of Myc-RF1-1-st-T at both mRNA and protein levels when NMD was active (Figure 5E, compare lanes 1 and 2 and Supplementary Figure S7B), but it failed to reduce Myc-RF1-1-st-T expressions in the presence of U1DN (Figure 5E, compare lanes 1 and 4). In fact, the Myc-RF1-1-st-T levels were significantly enhanced when NbRF1-3 and U1DN were co-expressed.

To exclude that heterologous expression results in an artifact, similar assays were conducted with *N. benthamiana* eRF1-1. The coding and terminator region of NbeRF1-1 was fused to a Myc tag (Myc-NbRF1-1-st-T), and then this construct was co-expressed in *N. benthamiana* leaves with NbRF1-1, with U1DN or with both constructs. As expected, co-expression of NbRF1-1 reduced Myc-NbRF1-1-st-T mRNA and protein levels when NMD was active (Supplementary Figure S7A and C), but it failed to decrease Myc-NbRF1-1-st-T levels when U1DN was co-expressed (Supplementary Figure S7A, compare lanes 1 and 4).

Interestingly, NMD inactivation led to more dramatic changes at protein than at mRNA levels. As Figure 5E and

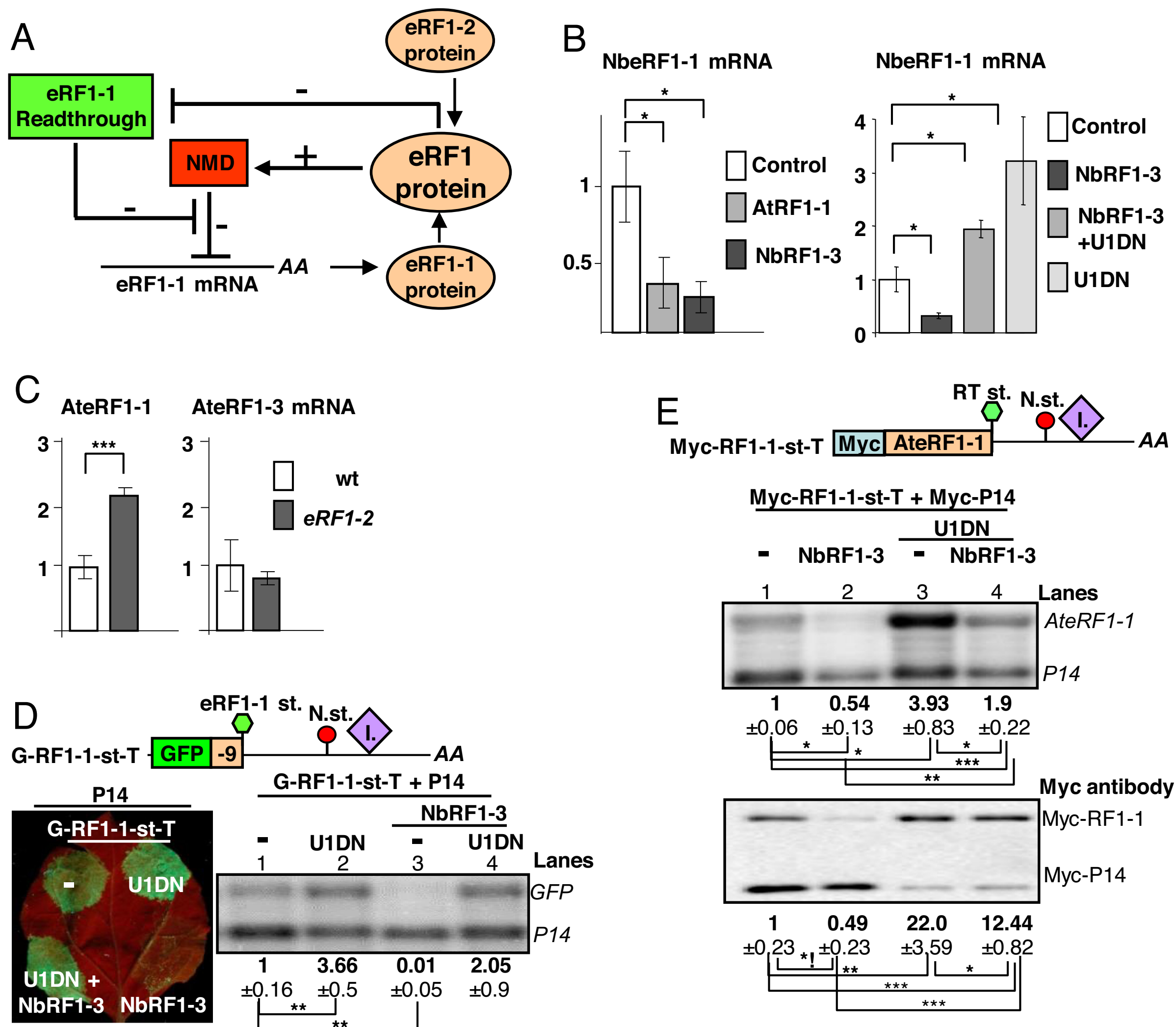

Figure 5. Autoregulation of eRF1. (**A**) Model of eRF1 autoregulation in plants. (**B**) eRF1 overexpression reduces the expression of eRF1-1 mRNA in an NMD-dependent manner. The eRF1-1 transcript level was measured by qRT-PCR in untreated *Nicotiana benthamiana* leaves (control) or in leaves in which *Arabidopsis* eRF1-1 or *N. benthamiana* eRF1-3 protein was overexpressed (left panel), or in leaves, in which the dominant-negative mutant of UPF1 (U1DN), the NbeRF1-3 protein or both U1DN+NbeRF1-3 were co-expressed (right panel). (**C**) eRF1-1 mRNAs are upregulated in *eRF1-2* mutant *Arabidopsis*. eRF1-1 and eRF1-3 mRNA levels were measured by qRT-PCR in wild-type (wt) and *eRF1-2* null mutant (*eRF1-2*) *Arabidopsis* lines. The assay was conducted and analyzed as described at Figure 2C. (**D**) eRF1 overexpression reduces the expression of eRF1-1 RT-NMD 3′UTR containing reporter transcripts in an NMD-dependent manner. G-RF1-1-st-T reporter was co-expressed with P14 (−), with P14+U1DN, with P14+NbRF1-3 or with P14+U1DN+NbRF1-3. The assay was conducted as described at Figure 2B. (**E**) eRF1 overexpression reduces the expression of the eRF1-1 protein in an NMD-dependent manner. The Myc-RF1-1st-T reporter gene, which contains the coding and the terminator region of *Arabidopsis* eRF1-1 fused to a Myc tag, was co-expressed with a Myc-tagged version of P14 internal control (Myc-P14), with Myc-P14+U1DN, with Myc-P14+NbRF1-3 or with Myc-P14+U1DN+NbRF1-3. The protein product of the Myc-RF1-1-st-T expresses to low levels in the Myc-RF1-1-st-T+Myc-P14 and the Myc-RF1-1-st-T+Myc-P14+NbRF1-3 samples (lanes 1 and 2), therefore three times more proteins were loaded from these samples onto the gel (to help visualization). To compare protein expressions, at each lane the signal of the Myc-RF1-1-st-T (upper band) was normalized to the corresponding Myc-P14 signal (bottom band). Mean values were calculated from three independent samples ($n = 3$, ± shows SD). The Myc-RF1-1-st-T/Myc-P14 ratio of the first sample (−) was taken as 1 and the Myc-RF1-1-st-T/Myc-P14 of the other samples are shown relative to it. The assay was conducted and analyzed as described at Figure 2B. Note that the co-expression of NbRF1-3 led to severely reduced Myc-RF1-1st-T protein expression. However, the effect was not significant at $n = 3$ (marked as*!). To clarify if these effects are significant, these parts of the experiment were repeated with six samples. These assays confirmed that co-expression of eRF1 proteins significantly reduces the protein expression of Myc-RF1-1st-T (see at Supplementary Figure S7B).

Supplementary Figure S7A show, co-expression of U1DN with Myc-RF1-1-st-T or Myc-NbRF1-1-st-T resulted in moderately increased transcript (3.93- and 2.41-fold) but strongly enhanced protein expression (22- and 14.3-fold increase).

eRF1 overexpression reduces the eRF1-1 levels in transgenic *N. benthamiana* plants

The predictions of autoregulatory model were also tested in transgenic plants. The coding and the terminator region of the *Arabidopsis* eRF1-1 was fused to a HA-tag (HA-RF1-1-st-T), and then transgenic *N. benthamiana* lines were gen-

erated with this construct, and with a control, in which the terminator region was replaced with the (canonical 3′UTR generating) 35S terminator (HA-RF1-1-35sT) (Figure 6A).

Several HA-RF1-1-st-T T_0 plants expressed the eRF1 transgenic protein to detectable levels. The transgenic protein expressed to moderate and variable levels in the different HA-RF1-1-st-T transgenic *N. benthamiana* plants (Figure 6A). T1 progenies of two transgenic lines (#6 and #7) were further studied. We assumed that in the T1 plants, the overall eRF1 protein level is enhanced and consequently the efficiency of translation termination is increased. Indeed, readthrough frequencies were significantly reduced in these lines. However, the eRF1 protein concentration still limited the efficiency of translation termination in these plants, as transient overexpression of NbRF1-3 further reduced the readthrough frequencies (Figure 6B). These data suggest that the functional eRF1 protein level is moderately enhanced in these transgenic plants. Increased eRF1 protein level is assumed to reduce the endogenous NbeRF1-1 mRNA expression. Indeed, NbeRF1-1 (but not the NbeRF1-2) mRNAs were down-regulated in the T1 progenies of the #6 HA-RF1-1-st-T transgenic line (Figure 6C).

Next we tested the influence of NMD inactivation and eRF1 overexpression on the expression of the endogenous NbeRF1-1 mRNA and on the levels of transgenic eRF1-1 transcript and protein. NbRF-1-3 protein, U1DN or both were expressed in the leaves of the T1 #6 HA-RF1-1-st-T plants and the endogenous NbeRF1-1 and transgenic eRF1-1 expressions were monitored. Relevantly, the transgenic eRF1-1 mRNA, the transgenic protein and the endogenous NbeRF1-1 mRNA levels were synchronously altered and the results were in line with the predictions of the autoregulatory model. We found that (i) U1DN expression enhanced the expression of the NbeRF1-1 mRNA and increased the transgenic eRF1-1 mRNA and protein levels (Figure 6D, left panels), (ii) overexpression of the NbeRF1-3 protein resulted in strongly reduced NbeRF1-1 mRNA and transgenic eRF1-1 mRNA and protein expressions (Figure 6D, middle panels) and that (iii) when U1DN was co-expressed with NbRF1-3 (Figure 6D, right panels), the transgenic and the NbeRF1-1 mRNA levels were enhanced. Thus, results obtained from transgenic *N. benthamiana* plants strongly support the eRF1 autoregulatory model.

We noted that overexpression of the NbeRF1-3 protein also reduced the level of NbeRF1-2 mRNA (Figure 6D, middle panels). However, this reduction was slight (0.75×) and the eRF1 overexpression did not diminish the NbeRF1-2 mRNA level in wild-type plants (Supplementary Figure S6A and B). By contrast, eRF1 overexpression led to severely decreased NbeRF1-1 mRNA levels in both wild-type and transgenic lines (0.2–0.4×). Thus, eRF1 protein effectively down-regulates the level of eRF1-1 transcript but only mildly (if at all) the eRF1-2 mRNA.

Although several HA-RF1-1-st-T T_0 plants were obtained, we could generate only one T_0 plant (#12) with the non-autoregulated HA-RF1-1-35sT construct. The #12 plant expressed the transgenic eRF1 protein to very high levels (Figure 6A) and it was sterile. These results suggest that strong overexpression of eRF1 protein is detrimental for plants. As expected, the endogenous NbeRF1-1 mRNA level was severely reduced in this plant (Supplementary Figure S8A). As the #12 plant was sterile, we could not study it further.

The HA-RF1-1-st-T construct was also used to generate transgenic *Arabidopsis* plants. Surprisingly, we could obtain only four T_1 transgenic *Arabidopsis* plants and only two of them (#2 and #4) expressed the transgenic eRF1-1 protein to detectable levels. In these lines, the transgenic mRNAs accumulated to high levels and were efficiently spliced (Supplementary Figure S8B). In line with the eRF1 autoregulation model, in the eRF1 transgenic protein expressing T_1 plants, the endogenous eRF1-1 mRNA levels were significantly reduced, while the eRF1-2 and eRF1-3 transcript levels were not altered (Supplementary Figure S8C). Unexpectedly, although the transgenic eRF1 protein still accumulated to easily detectable levels in the T_3 progenies, the expression of the endogenous eRF1-1 was similar to the wild-type plants (Supplementary Figure S8D). Although further studies are required to unravel the molecular basis of this unexpected finding, we hypothesizes, that eRF1 expression can be regulated by alternative ways and the eRF1-1 autoregulation is embedded into other regulatory circuits (see 'Discussion' section).

DISCUSSION

The autoregulatory circuit of plant eRF1

In order to maintain optimal translation termination efficiency, overall eRF1 protein concentration has to be finely regulated. We have shown that in plants, eRF1 protein level is regulated by a complex autoregulatory circuit that is based (i) on the special 3′UTR structure of eRF1-1-like mRNAs that can sense the eRF1 protein level, (ii) on NMD, which targets the eRF1-1-like transcripts and (iii) on readthrough, which protects these mRNAs from NMD (Figure 5A).

We found that eRF1 protein overexpression reduces the expression of eRF1-1-like but not eRF1-2-like transcripts (Figures 5B and 6; Supplementary Figure S6), while reduced eRF1 protein level leads to overexpression of eRF1-1-like but not eRF1-2-like mRNAs (Figure 5C). In addition, enhanced overall eRF1 protein level reduces the expression of the eRF1-1 protein (Figures 5E and 6D; Supplementary Figure S7A). Thus, the overall eRF1 level is negatively autoregulated in plants via regulation of eRF1-1 level. The results that eRF1-1 RT-NMD 3′UTR containing reporter mRNAs, like the endogenous eRF1-1 transcripts, are sensitive to eRF1 protein level (Figure 5D and Supplementary Figure S6A) indicate that, the RT-NMD 3′UTR structure senses eRF1 protein level.

The findings that enhanced expression of all three *N. benthamiana* eRF1 proteins inhibits readthrough and intensifies NMD in *N. benthamiana* leaves (Figure 4 and Supplementary Figure S5A) and that readthrough partially protects the eRF1-1 RT-NMD 3′UTR containing mRNAs from NMD-mediated decay (Figure 2A and B; Supplementary Figure S2B) explain how this 3′UTR structure can 'measure' the overall eRF1 protein level. As in plants, the efficiency of translational termination depends on eRF1 protein concentration (Figure 4), high eRF1 protein level

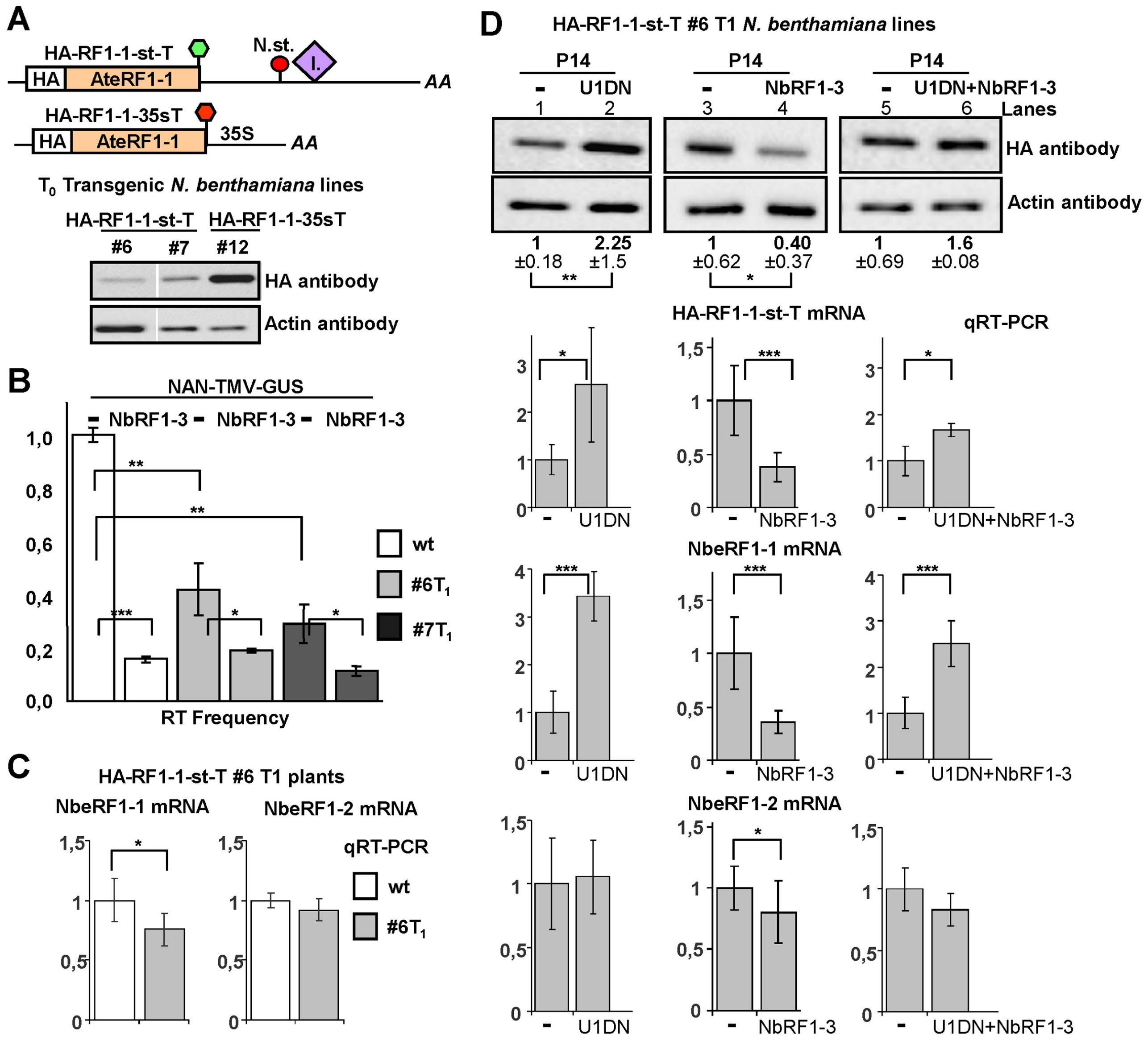

Figure 6. eRF1 overexpression reduces the eRF1-1 levels in transgenic *Nicotiana benthamiana* plants. (**A**) The HA-RF1-1-st-T reporter construct, in which the coding and the terminator region of the *Arabidopsis* eRF1-1 was fused to a HA tag and a control construct (HA-RF1-1-35sT), in which the coding region of *Arabidopsis* eRF1-1 gene with the 35S terminator was cloned to a HA tag, were transformed into *N. benthamiana* plants. HA western blot confirmed the transgene expression. Note that the transgene expression is much higher in the HA-RF1-1-35sT expressing T_0 line (#12) than in the HA-RF1-1-st-T transformed #6 and #7 lines. (**B**) The readthrough frequency is reduced in the HA-RF1-1-st-T transgenic plants. NAN-TMV-GUS readthrough reporter was co-infiltrated with P14 (–) or with P14+NbeRF1-3 (NbRF1-3) constructs into the leaves of wild-type (wt) or T_1 progenies ($n = 3$) of the #6 and #7 HA-RF1-1-st-T transgenic plants (#6T_1 and #7T_1). (**C**) The expression of the eRF1-1 but not the eRF1-2 mRNAs is reduced in the #6T_1 HA-RF1-1-st-T transgenic plants ($n = 6$). (**D**) eRF1 overexpression decreases the expression of both the transgenic and the endogenous eRF1-1 in an NMD dependent manner. Leaves of #6T_1 HA-RF1-1-st-T plants were infiltrated with P14 (–) or were co-infiltrated with P14 and a dominant-negative version of UPF1 (U1DN) or with NbRF1-3, or with both U1DN+NbRF1-3 ($n = 3$). HA western blot assays were conducted to monitor the transgenic eRF1-1 protein, while quantitative RT-PCR assays were carried out to follow the expression of the transgenic HA-RF1-1-st-T and the endogenous NbeRF1-1 and NbeRF1-2 mRNAs (upper, middle and bottom qRT-PCR panels).

leads to decreased occurrence of readthrough on the eRF1-1 mRNA, thereby enhancing the NMD sensitivity of the transcript. Moreover, high eRF1 protein level intensifies overall NMD. Thus, high eRF1 protein level destabilizes the eRF1-1 mRNA via NMD resulting in lower eRF1-1 and consequently, reduced total eRF1 protein concentration. Similarly, low eRF1 protein level leads to enhanced readthrough and weak NMD, which in turn results in increased eRF1-1 protein expression. As eRF1-1 transcript senses the total eRF1 protein level, this autoregulatory system stabilizes the total eRF1 protein concentration (Figure 5A).

The readthrough frequency is a critical element of the eRF1 autoregulation model. eRF1-1 readthrough should only partially rescue the 3′UTR induced NMD leaving an effective regulatory range to compensate for both reduced

and enhanced eRF1 protein expression. Indeed, we found that the moderate readthrough context is extremely conserved within the eRF1-1-like transcripts of seed plants (Supplementary Figure S1A and B) and that *Arabidopsis* and *N. benthamiana* eRF1-1 mRNAs are moderately overexpressed in NMD-deficient plants (Figure 2C and D, Supplementary Figure S6B and Supplementary Table S3).

Many of our results (Figures 5E and 6; Supplementary Figure S7A) suggest that the eRF1 autoregulatory circuit plays a role in buffering the enhanced eRF1 protein levels, thereby stabilizing the translation termination activity. However, although the endogenous eRF1-1 mRNA level was reduced in the T_1 HA-RF1-1-st-T transgenic *Arabidopsis* plants, the endogenous eRF1-1 mRNA expression was normal in the T_3 plants (Supplementary Figure S8D). This latter finding is seemingly inconsistent with the autoregulatory model. Interestingly, both the transgenic mRNA and protein levels were markedly increased in the T_3 relative to the T_1 plants (Supplementary Figure S8E). These data might suggest that while in the T_1 plants the eRF1 autoregulatory mechanism controls the expression of both the transgenic and the endogenous eRF1-1 mRNAs, in the T_3 plants the autoregulation does not function. We speculate that in the T_1 plants the efficiency of translation termination is increased due to the enhanced eRF1 protein concentration, and that the more intense termination reduces the expression of the endogenous eRF1-1 mRNA. We also hypothesize that in the T_3 plants, the efficacy of the translation termination is restored to the wild-type level as the very abundant transgenic eRF1 proteins accumulates in non-functional forms or because the efficiency of termination is limited by alternative factors (for example the eRF3 protein level is reduced in these lines). Alternatively, in wild-type *Arabidopsis*, unlike in *N. benthamiana*, the eRF1 protein level does not limit the efficiency of translation termination. In this case overexpression of eRF1 should not lead to more efficient termination and reduced eRF1-1 mRNA levels in the T_3 plants. However, if this assumption is correct, we do not know why the expression of the endogenous eRF1-1 mRNA is reduced in the T_1 plants. Further transgenic studies can clarify these issues.

The putative mechanism of eRF1 autoregulation

We propose a mechanistic model for how the eRF1 autoregulatory circuit could operate (Figure 7). Translation termination of the eRF1-1 mRNA should be inefficient because the stop codon is in a readthrough context and the 3′UTR harbors an EJC. Thus normal termination, readthrough and NMD might all occur at relatively high frequency (Figure 7). However, the fate of the mRNA is different depending on which one happens. (i) If translation is normally terminated at the readthrough stop, an eRF1-1 protein is released and the mRNA will undergo further rounds of translations. The transcript likely remains NMD-sensitive as the EJC would still be bound to the 3′UTR (Figure 7B). (ii) If slow termination activates NMD, it allows the release of the synthesized eRF1-1 protein but initiates the rapid decay of the eRF1-1 mRNA (Figure 7C). (iii) If readthrough occurs, the translation is terminated at the next stop and a C-terminally extended, perhaps non-functional (see below) eRF1-1 protein is generated. The termination at the next stop is efficient, thus the mRNA remains stable. We propose that during the readthrough-mediated elongation the translating ribosome removes the EJC from the 3′UTR, thereby generating an NMD-insensitive eRF1-1 mRNA that can undergo further rounds of translation and produce normal eRF1-1 proteins (Figure 7D).

As the C-terminally extended readthrough protein product of eRF1-1 was non-functional in translation termination assays (Supplementary Figures S4C and S5C) and because the extended regions are not conserved and do not contain protein motifs (Supplementary Figure S9), we propose that the extended eRF1-1 protein is biologically inactive. Thus readthrough might function to eliminate EJC, instead of producing a C-terminally extended eRF1-1 protein.

Interestingly, even relatively low level of readthrough can significantly protect an mRNA from NMD in yeast, mammals (30,31) as well as in plants (this study). We hypothesize that readthrough assays measure the readthrough frequency during the bulk translation, while it could be significantly higher during the first, pioneer round of translation, which is different from the bulk translation in many aspects (56). Relevantly, if readthrough occurs on the eRF1-1 mRNA during the pioneer translation, the eRF1-1 transcript will be EJC-free, thus it could be efficiently translated during the bulk translation (Figure 7D).

Evolution of eRF1 autoregulatory circuit

Negative autoregulation stabilizes gene expression by reducing noise and accelerating the restoration of optimal expression levels after fluctuations (57). RF2 expression is negatively autoregulated in prokaryotes. RF2 transcripts contain an early stop codon in a context that facilitates +1 frameshift. High RF2 level leads to frequent termination at the early stop codon, while low RF2 level results in frequent +1 frameshifts and efficient generation of functional RF2 (9). The early stop codon in the frameshift context makes the transcript especially sensitive to RF2 level, even slight reduction of RF2, which might not alter termination efficiency at normal stop contexts, leads to enhanced frameshift and increased RF2 synthesis. The giant viruses of *Acanthamoeba* (mimivirus and megavirus) might also encode an autoregulated termination factor. It is proposed that the production of the RF1 proteins of the giant viruses requires two recoding events, a frameshift and a readthrough, and that the frequency of both events depends on the concentration of the RF1 protein (58). Our results suggest that eRF1 autoregulation also evolved in plants. Thus we propose that negative autoregulation of the key termination factor convergently evolved at least three times. However, in bacteria and giant viruses the functional termination factor is generated by the relatively rare recoding event, whereas in plants the functional eRF1-1 protein is produced by the normal termination event.

As eRF1-1-like transcripts are present in all angiosperms, and because *Picea abies*, a gymnosperm whose genome is sequenced, expresses two eRF1 transcripts, both with an intronic RT-NMD 3′UTR (Supplementary Table S2), we propose that the complex eRF1 autoregulatory circuit already operated in the common ancestor of seed plants (the evo-

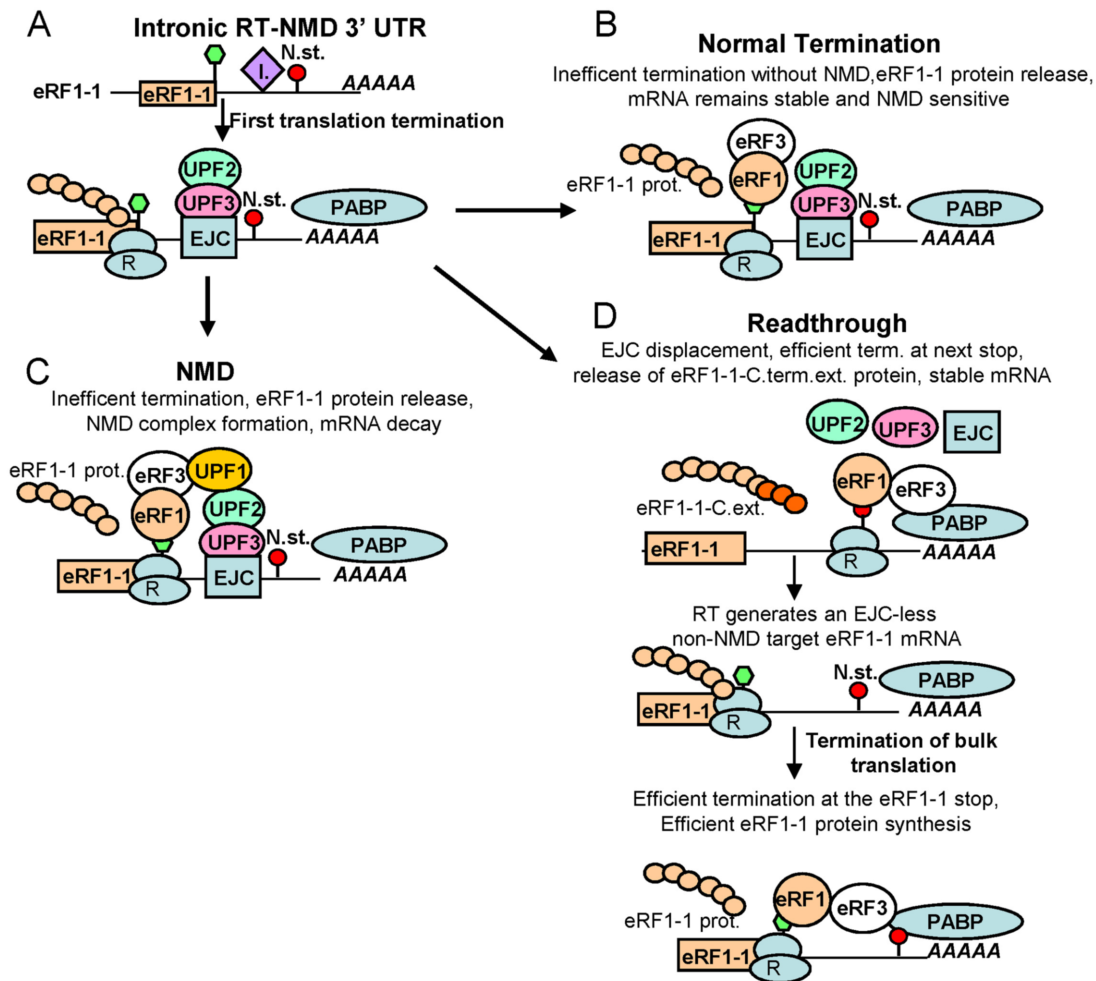

Figure 7. The putative mechanism of eRF1 autoregulatory control.

lution of eRF1 autoregulation within *Plantae* supergroup is further discussed at Supplementary Figure S10). Negative autoregulation might facilitate gene duplication by attenuating the gene dosage effect. Thus the eRF1-RT-NMD autoregulatory circuit could facilitate the fixation of eRF1 duplicates. Moreover, one copy can lose the homeostatic *cis* element if the autoregulated copy can compensate for fluctuations in the expression of both copies (59). Indeed, in angiosperms eRF1 is present in multiple copies, but at least one of them encodes an eRF1 mRNA with an autoregulated, intronic RT-NMD 3′UTR (Supplementary Table S2 and Supplementary Figure S10).

The eRF1 autoregulatory circuit might act as a homeostatic control system to stabilize the intensity of readthrough, NMD and translation termination

Our data suggests that in plants, the intensity of all three types of termination coupled events depend on eRF1 protein concentration. eRF1 level is a limiting factor for efficient translation termination and NMD, and negatively correlates with readthrough frequency. In theory, the eRF1 autoregulatory system (in addition to stabilizing eRF1 protein level) could act as a homeostatic control mechanism that compensates for changes in the intensity of NMD or readthrough. For instance, more intense NMD leads to decreased eRF1-1 expression, which in turn results in reduced NMD activity. Similarly, increased readthrough, which might be the consequence of enhanced near cognate tRNA expression, protects the eRF1-1 transcript from NMD more efficiently. Thus increased readthrough leads to enhanced eRF1 protein expression, which in turn reduces the intensity of readthrough.

As the eRF1-1 autoregulatory circuit might buffer fluctuations in eRF1 protein level, NMD and readthrough, we propose that this complex regulatory system helps to keep the balance between efficient translation termination, readthrough and NMD.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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