Final report: Regulation of translation termination in plants

The original grant (K 116963) was transferred, and then it was continued under a new grant number (K 136512).

The background and the aims of the program

Eukaryotic translation consists of three main steps, initiation, elongation and termination (including recycling). Previously it was assumed that initiation is the regulated phase of translation. However recent results have demonstrated that elongation and termination could also be strictly controlled.

Termination is the final step of translation. Binding of the eukaryotic Release Factor 1 (eRF1) in a complex with the eRF3 protein to the stop codon at the A-site stimulates peptide release and the disassembling-recycling of the ribosome. Infrequently, if a transcript contains a specific signal or the conditions do not favor the efficient translation termination, alternative events such as translational readthrough or Nonsense-mediated decay (NMD) can occur at the stop codon. At readthrough, a near cognate tRNA binds to the stop codon, thus the elongation is continued till the next in-frame stop codon. NMD is a translation termination coupled eukaryotic quality control system that identifies and degrades aberrant transcripts having unusual 3' untranslated region (3'UTR). Previously we have shown that plant NMD recognizes a mRNA as an aberrant one (and initiates its decay) if the 3'UTR of the transcript is unusually long or contains an intron more than 50 nt downstream from the stop codon (Kertész et al., 2006; Nyikó et al., 2013). If translation elongation stops in the coding region the eRF1-eRF3 cannot terminate translation as a coding codon is present at the A-site. In this situation quality control systems are activated, which degrades the aberrant transcript and allows the recycling of the ribosome.

Although the last step of translation has been intensively studied in yeast and animals, very **little was known about translation termination in plants.** Plant **eRF3 was not identified** and **eRF1 was** only **poorly characterized**. However, it was **shown that** in the model dicot organism *Arabidopsis thaliana* eRF1 is present in three copies and that, in plants, like in yeast, both **over- and underexpression of eRF1 lead to altered phenotype**. Moreover, we have observed that in *Arabidopsis* **NMD** a translation termination coupled quality control system **regulates** the key translation termination factor **eRF1**. Based on these observations **we hypothesized that translation termination can be specifically regulated in plants.**

During this program we wanted to understand the regulation of translation termination in plants. We identified and **characterized plant eRF3** (Auber et al., 2018). By combining various transient assays and transgenic approaches we **unraveled that** in plants **eRF1** expression **is controlled by a sophisticated autoregulatory circuit** in which both termination coupled events, readthrough and NMD are involved (Nyikó et al., 2017). We showed that this specific autoregulatory system **has covergently evolved** at least twice in eukaryotes, once **in flowering plants and** once **in fungi** (Kurilla et al., 2020). Finally we have **analyzed** the alternative translation termination events such as **No-go decay system that can terminate translation independently of stop codons** (Sulkowska et al., 2020; Szádeczky-Kardoss, Csorba, et al., 2018; Szádeczky-Kardoss, Gál, et al., 2018). Interestingly we found that **this quality control system acts cooperatively with the RNA silencing** system, suggesting that these alternative translation termination mechanisms are required for small RNA and mRNA homeostasis (Szádeczky-Kardoss, Csorba, et al., 2018; Auth, 2021, submitted manuscript).

We think that **our results significantly contributed to our understanding about the mechanism, regulation and evolution of plant translation termination.**

Characterization of plant eRF3

Surprisingly, when we started the project the eRF3 translation termination factor was not described in plants. Rhus we have identified and briefly characterized plant eRF3. These results were published in a short paper (Auber et al., 2018).

It was previously suggested that AT1G18070 encodes the eRF3 protein in *Arabidopsis*. Indeed, the predicted protein shows strong similarity to the yeast and mammalian eRF3s. The C-terminal regions are highly conserved, while the disordered N-terminal regions show weak sequence conservation even within plants.

We failed to significantly down regulate the putative eRF3, and its overexpression did not modify the efficiency of translation termination. Therefore we tried to indirectly prove that AT1G18070 encodes a functional eRF3. As eRF3 forms a strong heterodimer with eRF1 in eukaryotes, we postulated that if AT1G18070 encodes a functional eRF3, it will bind eRF1. Indeed, co-immunoprecipitation assays confirmed that the putative eRF3 protein interacts with plant eRF1 (Fig.1). Thus we concluded that AT1G18070 encodes a functional eRF3. Next we studied the regulation of eRF3 expression.

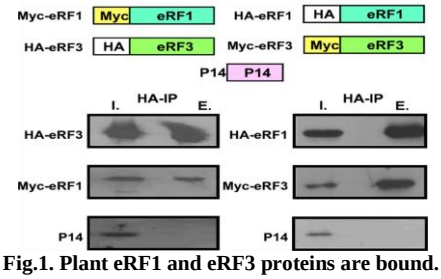

Fig.1. Plant eRF1 and eRF3 proteins are bound.

We have shown that eRF1 and eRF3 are present in physiologically different concentrations, increased eRF3 level does not modify the efficiency of termination, while increased eRF1 concentration leads to more efficient termination and to reduced readthrough frequency.
eRF3, unlike eRF1 (see below), is not autoregulated, eRF3 overexpression did not result in reduced endogenous eRF3 mRNA levels. However, we found that the expression of the eRF1 and eRF3 translation termination factors are connected in plants, eRF1 transcript levels were altered in eRF3 overexpressing tissues, while eRF3 mRNA levels were modified in eRF1 underexpressing leaves (Fig.2).

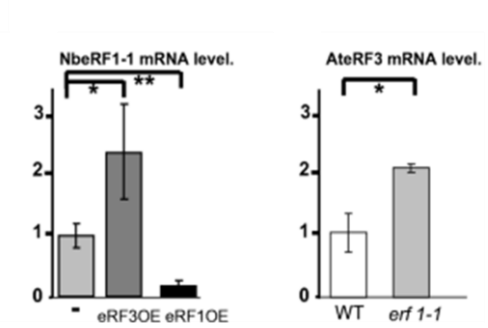

Fig.2. eRF3 and eRF1 expressions are connected. eRF3OE and eRF1OE show eRF3 and eRF1 overexpressing plants, respectively. *erf1-1* is a mutant line.

Regulation of eRF3 translation termination factor is also linked to NMD quality control system. According to the current model, if translation termination is inefficient, eRF3 recruits UPF1 NMD factor to the terminating ribosome, which then initiates the decay of the transcript. In line with this model we found that plant eRF3 (but not eRF1) can be co-immunoprecipitated with UPF1. Moreover, our data suggest that in certain plants such as tobacco NMD directly regulates eRF3 expression. In tobacco eRF3 is present in two copies, and one of them expresses a mRNA that is directly targeted by NMD. This NMD sensitive eRF3 mRNA has two NMD inducing features, its 3'UTR is unusually long and it contains an NMD inducing upstream ORF in the 5'UTR region. Finally we found that NMD might act as a conserved, highly sensitive fail-safe system to prevent accumulation of truncated eRF3 proteins in plants. In all angiosperms, eRF3 transcripts contain an intron in the 3'UTR region, which is too close to the stop codon to induce NMD (Fig.3). Why is it conserved? We speculate that accumulation of a slightly truncated eRF3 protein would be detrimental, it could significantly reduce the efficacy of termination. This conserved intron prevents these events. If a mutation generates a premature stop codon close to the normal stop, the intron will be present in NMD inducing position (more than 50 nt form the premature termination codon), thus the transcript will be targeted-degraded by the NMD system and the potentially detrimental C-terminally truncated eRF3 cannot accumulate.

Fig.3. NMD is a sensitive fail-safe system to prevent the accumulation of slightly truncated eRF3 proteins. I. Indicates intron, PTC shows premature termination codon.

eRF1 expression is regulated by a complex autoregulatory circuit in plants
eRF1 is the key eukaryotic translation termination factor, it binds to the stop codon, facilitates peptide release and promotes ribosome splitting-recycling. At the beginning of this project, it was known that both over- and underexpression of eRF1 lead to growth phenotype in plants. Moreover, it was demonstrated that while in most eukaryotes eRF1 is present as a single copy gene, in *Arabidopsis* model plant eRF1 is present in three copies. Thus we hypothesized that eRF1 expression should be especially strictly controlled in plants. Indeed, our data confirmed that a very specific autoregulatory circuit, in which both readthrough and NMD alternative translation termination events play a critical role, regulates the expression of eRF1 in plants. eRF1 autoregulation was described in a paper published in Nucleic Acids Research (Nyikó et al., 2017).
We found that although the three *Arabidopsis* eRF1 genes (eRF1-1, eRF1-2 and eRF1-3) encode very similar eRF1 proteins, their transcripts are markedly different (Fig.4). While eRF1-2 and eRF1-3 mRNAs have normal 3'UTRs, the eRF1-1 mRNA has a very specific 3'UTR structure. We named this unique 3'UTR as RT-NMD (readthrough-NMD) 3'UTR structure. A mRNA has an RT-NMD 3'UTR structure if (1) it contains an intron in the 3'UTR in a NMD inducing position (more than 50 nt from the stop codon), (2) the stop codon of the transcript is in a translational readthrough facilitating sequence context, and if (3) the next in-frame stop (next stop) is present in a non-NMD activating position (located close to or downstream the 3'UTR intron). RT-NMD 3'UTR is extremely rare in plants, in *Arabidopsis* eRF1-1 is the only RT-NMD 3'UTR containing transcript. Relevantly, we showed that in all Angiosperms eRF1 is present in multiple copies and that at least one copy expresses an RT-NMD 3'UTR structure containing eRF1-1 like transcript.

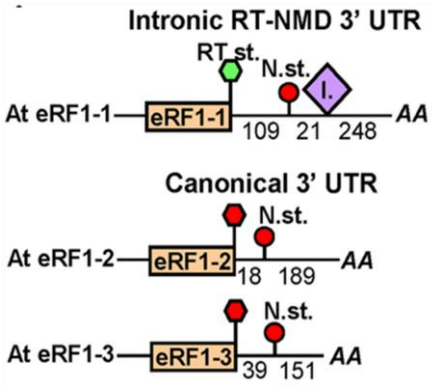

Fig. 4. *Arabidopsis* eRF1 transcripts. eRF1-1 mRNA has an RT-NMD 3'UTR structure. Red and green hexagons show stop codons in normal and readthrough (RT) context.N.st. indicates the next in-frame stop codon, I. shows the NMD inducing intron. Numbers show the distance in nucleotides.

Using various transient assay systems, we demonstrated that eRF1 protein level is autoregulated in plants, and that, this autoregulation is based on the specific RT-NMD 3'UTR structure of eRF1-1 mRNA. We found that (1) overexpression of any eRF1 protein leads to reduced eRF1-1 transcript level (while eRF1-2 and eRF1-3 mRNA levels are not altered), and that (2) reduced eRF1 level results in enhanced eRF1-1 (but not eRF1-2 and eRF1-3) mRNA expression. Using transgenic plants, we could also demonstrate that eRF1-1 protein levels are strongly correlated with the mRNA levels. We proved that RT-NMD 3'UTR structure was required for this autoregulation, the eRF1-1 transcript was not sensitive to the eRF1 protein level if the RT stop codon context is impaired, if the 3'UTR intron is eliminated or if the position of the next stop codon is modified.
We have unraveled the molecular basis of eRF1 autoregulatory circuit. Our data suggest that the different translation termination related events are interconnected in plants. We demonstrated that enhanced eRF1 protein level leads to more efficient translation termination and consequently to reduced readthrough frequency in plants. Moreover, the intensity of plant NMD also depends on eRF1 concentration, the higher the eRF1 level, the more intense the NMD. Finally, we found that readthrough can interfere with plant NMD. We demonstrated that readthrough can partially rescue the mRNA from the 3' UTR intron activated NMD if the next stop is not in a NMD inducing position. We proved that the RT-NMD 3' UTR structure of eRF1-1 mRNA and these complex connections allow eRF1 autoregulation.
The eRF1 autoregulation model (Fig.5): eRF1-1 mRNA is a moderate target of plant NMD because readthrough of the eRF1-1 mRNA partially protects the transcript from its 3'UTR induced NMD. If eRF1 protein level is increased, the readthrough frequency at the eRF1-1 mRNA stop codon will be reduced, while the general NMD intensity is increased. Thus eRF1-1 mRNA will be more efficiently degraded by NMD, which leads to reduced eRF1-1 protein production and consequently restores the total eRF1 protein level. Similarly, if the total eRF1 protein concentration is low, NMD will be weak and the readthrough at eRF1-1 stop codon will be efficient. Thus eRF1-1 transcript level will be protected from NMD and the enhanced eRF1-1 mRNA level leads to increased eRF1-1 protein production and enhanced total eRF1 protein level.

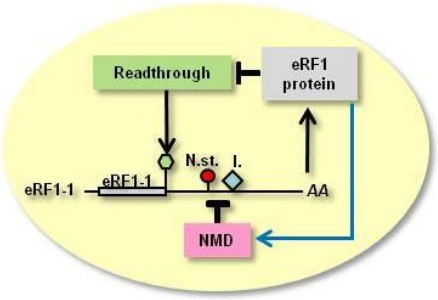

Fig. 5. eRF1 autoregulation. If total eRF1 protein level is enhanced, readthrough frequency will be low at eRF1-1 stop codon, while NMD is intensified. Thus NMD will target eRF1-1 transcripts more efficiently leading to reduced eRF1 protein expression. Blue line shows plant specific connection. Other connections also operate in *Neurospora* (see below).

We propose that this eRF1 autoregulatory system stabilizes the eRF1 protein level. Moreover it is tempting to speculate that this circuit might ensure that the three translation termination related events, normal termination, readthrough and NMD can act in a fine balance in plants. For instance, if readthrough is unusually intense (by overexpressing a near cognate tRNA), eRF1-1 mRNA will be more efficiently protected from NMD, thus eRF1 protein level is increased and high eRF1 level leads to reduced readthrough frequency. Similarly, weak NMD leads to enhanced eRF1 protein level, which intensifies NMD.

Evolution of eRF1 autoregulation
We demonstrated that a sophisticated autoregulatory circuit regulates eRF1 level in plants. As RT-NMD structure is extremely rare and because eRF1 autoregulation depends on the RT-NMD structure of eRF1 mRNA, we postulated that if any organism

has eRF1 mRNA with RT-NMD structure, it is autoregulated. Thus to understand the evolution of eRF1 autoregulation we comparatively studied the 3'UTR structure of eRF1 mRNAs in different eukaryotes. Our results related to the evolution of eRF1 autoregulation were published in part in the plant eRF1 paper (Nyikó et al., 2017) and in a Febs Letters manuscript, in which we described the mechanism of eRF1 autoregulation in *Neurospora crassa* and studied the evolution of the eRF1 autoregulatory circuit.

We found that all Angiosperms and Gymnosperms contain multiple eRF1 copies and that at least one of them always has an RT-NMD structure. By contrast, RT-NMD eRF1 mRNA structure has not been found in mosses and green algae. Thus we conclude that the eRF1 autoregulatory circuit was already present in the common ancestor of Gymno- and Angiosperms and that strong negative selection prevents loss of autoregulation in higher plants (Nyikó et al., 2017). Surprisingly, we found that certain fungi including the *N. crassa* (Fig.6) model organism also contain RT-NMD eRF1 transcripts (Kurilla et al., 2020).

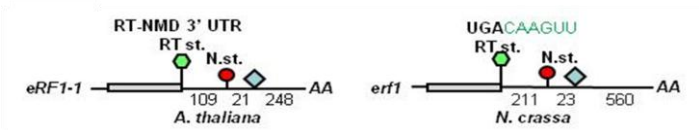

Fig.6. *Arabidopsis* eRF1-1 and *Neurospora* erf1 transcripts have similar RT-NMD 3'UTR structure.

Confirming that eRF1 RT-NMD structure is a good indication of autoregulation, we demonstrated that eRF1 is also autoregulated in *N. crassa.* eRF1 overexpression without the autoregulatory RT-NMD 3'UTR structure leads to slower growth suggesting that eRF1 autoregulation is physiologically relevant in *Neurospora*. eRF1 autoregulation is mechanistically similar in plants and *Neurospora* except that in the latter organism enhanced eRF1 protein level does not lead to more intense NMD (Fig.5). It suggests that this element is not essential for the autoregulatory circuit. We identified RT-NMD eRF1 3'UTR in Mucoromycota phylum and in both phyla (Ascomycota and Basidiomycota) of the Dikarya subkingdom, while it was not found in the early-diverging fungal phyla. As RT-NMD eRF1 transcripts were not found in other eukaryotic branches, we propose that eRF1 RT-NMD 3'UTR-based eRF1 autoregulatory circuit has convergently evolved twice in eukaryotes, once in the ancestor of higher plants and once in fungi. eRF1 autoregulation is more conserved in plants than in fungi. RT-NMD containing eRF1-1 is present in all plants, while many branches of Dikarya and Mucoromycota (including yeasts or fission yeasts) contain a single eRF1 gene without RT-NMD structure. Why RT-NMD structure of eRF1 is so easily lost in fungi but never in plants? While eRF1 is a single copy gene in most fungi, it is always present in multiple copies in plants. As multiple eRF1 copies could lead to more fluctuating expression, autoregulation based stabilization of eRF1 level is more important in plants. It is also possible that alternative eRF1 regulatory systems act more efficiently in fungi, hence loss of RT-NMD 3'UTR-based eRF1 autoregulation is more tolerable.

The expression of the key translation termination factor should be finely adjusted as both low and high levels can modify the intensity and fidelity of translation. Autoregulation is an efficient 'solution' to stabilize the expression of translation termination factors. However, these factors are required for the expression of all protein coding genes, thus for efficient autoregulation, they have to contain specific sensitizing elements that make them especially sensitive to the concentration of termination factor. In prokaryotes, the RF2 translation termination factor can be autoregulated as the RF2 mRNA contains a sensitizing element, an early stop codon in a frameshift context. Low RF2 results in frequent frameshift at the early stop codon and efficient synthesis of the functional RF2 protein, while high RF2 leads to frequent termination at the early stop codon. In prokaryotes the functional translation termination factor is generated by stop codon recoding (frameshift) events. As recoding is much less efficient in eukaryotes, recoding-based eRF1 autoregulation would not be functional in eukaryotes. Indeed, although eRF1 is also autoregulated in plants and fungi, in these eukaryotes the functional eRF1 protein is generated during normal translational termination (instead of recoding) and the RT-NMD 3'UTR could act as sensitizing element.

We believe that it is a "text book" example of convergent molecular evolution. Different mechanisms have evolved in prokaryotes and eukaryotes to solve the same challenge, how the mRNAs of a translation termination factor could be more sensitive to the level of the corresponding termination factor than any other transcripts. In prokaryotes a frameshift "sensing" sequence evolved, while in eukaryotes an RT-NMD 3'UTR structure evolved. Interestingly, the RT-NMD 3'UTR based eRF1 autoregulation evolved at least twice in eukaryotes.

Paralogs of translation termination factors are involved in different RNA quality control

At the beginning of the program it was already known that many eukaryotes contain paralogs of translation termination factors. Pelota (Dom34 in yeast) and HBS1 are similar to eRF1 and eRF3, respectively. It was also known that these proteins can complete translation in the absence of a stop codon and that they play role in the elimination of different aberrant transcripts. To clarify their role in plant translation termination we searched for homologs of Pelota and HBS1 in *Arabidopsis* and studied the function of identified genes. We have shown that Pelota is present in two copies in *Arabidopsis*, one is a constitutively expressed functional gene (Pelota), while the second encodes a dominant-negative mutant protein (Pel2), which is expressed only in the seeds. We demonstrated that Pelota and HBS1 are involved in Non-stop decay (NSD), No-go decay (NGD) and RNA silencing (but not in NMD) RNA quality control systems in plants. Our data suggest that the Pelota-HBS1 complex terminates translation in plants in the absence of stop codon. Our results related to the function of Pelota-HBS1 alternative translation termination system were described in papers published in Nucleic Acids Research, Plant Science (Szádeczky-

Kardoss, Csorba, et al., 2018; Szádeczky-Kardoss, Gál, et al., 2018) and in a manuscript that is under review at Plant Molecular Biology (Auth et al., 2021).
Although NSD and NGD was regarded at the beginning of the project as independent systems, recent results including our own plant data suggest that the two quality control systems are strongly connected (see below). NSD identifies and degrades aberrant transcripts, which contains a polyA tail but lacks an in-frame stop codon (called nonstop mRNAs). These faulty mRNAs are frequently generated if premature polyadenylation occurs in the codon region. We showed that these nonstop transcripts are dramatically overaccumulated in the absence of Pelota, HBS1 or when Pel2 is overexpressed, and that the mRNAs accumulate in deadenylated forms. No-go decay quality control system recognizes and degrades transcripts that contain translation elongation blocking sequences. If the translating ribosome is permanently stalled, NGD cleaves the transcript thereby preventing further translation of the faulty mRNA, terminates translation and facilitates ribosome recycling.
We demonstrated that NGD also functions in plants and identified the *cis* and t*rans* factors of plant NGD. In yeast NGD can be activated by various *cis* elements, such as rare codons, basic amino acid stretches, long A-sequences, stem-loop structures. We found that in plants only A-stretches and less efficiently stem-loops can activate NGD. A-stretches induce plant NGD in length and position dependent manner, the longer the A-stretch the more efficient the NGD mediated cleavage. We show that NGD activating A-stretch is not present in the coding regions of *Arabidopsis* genome. Thus we propose that plant NGD is only activated on non-stop transcript. When the elongating ribosome (in the absence of the in-frame stop codon) reaches the polyA sequence it stalls and induces NGD, which cleaves the transcript. We showed that NGD mediated cleavage occurs upstream of the blocking A-stretch and that it functions in a position dependent manner. The A-stretch should be located at least 90 nt from the start codon to trigger efficient NGD cleavage.
We have also studied the *trans* factors of NGD. Although the plant NGD endonuclease is still not known, we could identify couple of factors that are involved in the elimination of NGD cleavage fragments. We demonstrated that Pelota and HBS1 are not essential for the cleavage but they are required for the elimination of the 5' NGD cleavage fragments. Moreover we showed that the SKI-exosome conserved eukaryotic 3'-5' exonuclease system degrades these 5' NGD cleavage products. Our data suggest that after cleavage the next ribosome runs to the 3' end of the 5' cleavage fragment. When it reaches the end, the P site is occupied with the tRNA linked the peptide, while the A-site is empty. It is likely that the Pelota-HBS1 complex bounds to the empty A-site, terminates translation, rescues the ribosome and recruits the SKI-exosome to degrade the mRNA. We showed that RST1 and RIPR plant specific proteins are also required for the elimination of the NGD 5' cleavage fragments. Our preliminary results suggest that in plants these proteins link the SKI and exosome complexes (in other eukaryotes sKI7 links SKI and exosome complexes). We showed that the 3' NGD cleavage fragment is decayed by the XRN4 exonuclease in plants.
Most interestingly we found that NGD cooperates with the RNA silencing system. In plants, microRNAs regulate transcripts by triggering the cleavage of the target transcripts in the coding region. We showed that the Pelota-HBS NGD complex, the RST1, RIPR proteins and the SKI-exosome complexes are required for the degradation of the 5'cleavage fragments of micro RNA cleaved plant transcripts. Our data at least partially explains why microRNA cleaved transcripts are not subjected for further silencing amplification. When viral RNAs are cleaved by the silencing machinery, RDRs (RNA-dependent RNA polymerase) generate double stranded RNAs efficiently from the cleavage fragments. These dsRNAs are sources of new small RNAs, which play a role in antiviral defense. Silencing amplification is essential for efficient antiviral defense. It is not known why miRNA cleaved endogen transcripts are not subjected to silencing amplification. Our data partially explains it, if endogen transcripts are cleaved, the Pelota-HBS, RST1-RITS, SKI-exosome system quickly eliminates the 5' cleavage fragments. In the absence of these systems, silencing amplification could generate detrimental secondary small RNAs from microRNA cleaved transcript. Thus our data show that different RNA quality control systems are connected in plants and that it is essential to maintain the normal small RNA and mRNA homeostasis.

Collaborations
Most of the experiments were conducted in our lab and in all papers (except the Sulkowska et al., 2020) the first and the corresponding authors belong to our lab. However, during this program we fruitfully collaborated with many different groups, without their contribution many valuable parts of the program could not be completed. Thanks for all of them. We cooperated with the Nagy group on eRF1 autoregulation in plants (Nyikó et al., 2017), with the Burgyan, Csorba, Taller and Orban groups on NSD and silencing program (Szádeczky-Kardoss, Csorba, et al., 2018), with the Taller group on NGD (Szádeczky-Kardoss, Gál, et al., 2018), with the Kaldi group on *Neurospor*a eRF1 (Kurilla et al., 2020) and with the Kufel group on NMD (Sulkowska et al., 2020).

Papers **(the title of the papers that are linked to the program are in bold)**

Auber, A., Nyikó, T., Mérai, Z., & Silhavy, D. (2018). **Characterization of Eukaryotic Release Factor 3 (eRF3) Translation Termination Factor in Plants.** *Plant Molecular Biology Reporter*, *36*(5–6), 858–869. https://doi.org/10.1007/s11105-018-1128-5

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Kurilla, A., Szőke, A., Auber, A., Káldi, K., & Silhavy, D. (2020). **Expression of the translation termination factor eRF1 is autoregulated by translational readthrough and 3'UTR intron-mediated NMD in Neurospora crassa.** *FEBS Letters*. https://doi.org/10.1002/1873-3468.13918

Nyikó, T., Auber, A., Szabadkai, L., Benkovics, A., Auth, M., Mérai, Z., Kerényi, Z., Dinnyés, A., Nagy, F., & Silhavy, D. (2017). **Expression of the eRF1 translation termination factor is controlled by an autoregulatory circuit involving readthrough and nonsense-mediated decay in plants.** *Nucleic Acids Research*, *45*(7), 4174–4188. https://doi.org/10.1093/nar/gkw1303

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Szádeczky-Kardoss, I., Csorba, T., Auber, A., Schamberger, A., Nyikó, T., Taller, J., Orbán, T. I., Burgyán, J., & Silhavy, D. (2018). **The nonstop decay and the RNA silencing systems operate cooperatively in plants.** *Nucleic Acids Research*, *46*(9), 4632–4648. https://doi.org/10.1093/nar/gky279

Szádeczky-Kardoss, I., Gál, L., Auber, A., Taller, J., & Silhavy, D. (2018). **The No-go decay system degrades plant mRNAs that contain a long A-stretch in the coding region.** *Plant Science*, *275*, 19–27. https://doi.org/10.1016/j.plantsci.2018.07.008