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Investigation of translation-coupled mRNA quality control systems in plants

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INTRODUCTION AND OBJECTIVES

Eukaryotic gene expression is a tightly regulated process, an important element of which is the regulation of mRNA composition. The mRNA composition of a cell is determined by the balance of mRNA synthesis and degradation. To keep mRNA homeostasis, various mRNA degradation processes, like normal mRNA degradation, quality control and silencing systems should act in balance. When this balance is disrupted, it leads to severe growth and developmental disorders in plants, therefore it is important to get to know the operation and regulation of different RNA degradation systems, as well as to map the connections between them. In this field, our group mainly studies the operation and regulation of plant RNA quality control systems. During my work, I also examined the operation or biological regulatory effect of different plant RNA quality control systems.

In plants aberrant mRNAs recognized and degraded by different RNA quality control systems. Various aberrant mRNAs produced during transcription and RNA processing. The accumulation of truncated proteins translated from these aberrant mRNAs can be detrimental to the cell, therefore rapid and efficient degradation of defective mRNAs and their protein products, as well as ribosome disassembly and recycling is essential. The Nonsense-mediated mRNA decay (NMD) system is involved in detection and decay of mRNA transcripts which contain premature termination codons (PTCs). The No-Go decay (NGD) degrades faulty mRNAs harbouring an element that blocks the elongation step of translation. The Non-Stop decay (NSD) eliminates aberrant transcript lacking an in-frame stop codon.

In addition to its quality control function, NMD regulates the expression of several physiological mRNAs. NMD is a translation-coupled quality control system that is especially related to the termination step of translation. Inefficient termination can lead to translational stop codon readthrough (RT) or NMD. RT and NMD are physiologically important, while proper translation termination is

essential in all eukaryotes. As the eukaryotic Release Factor 1 (eRF1) is the key factor of translation termination, strict control of eRF1 protein level is essential for efficient gene expression. However, little is known about the regulation of eRF1 expression. Based on previous results, our group assumed that the eRF1 level is regulated by a complex autoregulatory circuit, in which NMD plays a critical role. These observations suggest that in plants, the eRF1 level can be autoregulated and this self-regulation is based on the eRF1 sensitivity of the specific structure of eRF1-1 mRNA. They proposed a mechanistic model for how the eRF1 autoregulatory circuit could operate. Our group previously verified each element of this model at mRNA level. However, in the absence of an appropriate endogenous eRF1-1 antibody, the validity of the model at the protein level has not been directly investigated so far.

The termination step of translation is also inhibited when the elongation is blocked or the transcript lacking an in-frame stop codon. These faulty transcripts are recognized by the NGD or NSD translation-coupled mRNA quality control systems, which degrade the aberrant transcript and also ensure ribosome recycling. Although the core trans-acting NGD and NSD factors are conserved in eukaryotes, the cis-acting elements may be various in different organisms. Our group have identified the plant NGD trans-acting factors and demonstrated that in plants only long A-stretches can cause ribosome stalling and induce NGD efficiently. It is known that in yeast, in addition to the quality of the blocking sequence, its position is also crucial in NGD activation.

Ribosome stalling can occur not only on the main ORF (open reading frame) but also on ORFs in the 5'UTR (untranslated region). Degradome studies reveal that truncated mRNAs with ribosomes stalled on them are enriched among uORF (upstream ORF) containing transcripts. However, in most cases, the role of RNA quality control systems in the degradation of these mRNAs has not been clarified to date. Some of the uORF-bearing transcripts are subjected to NMD

degradation. However, it has been suggested that uORFs that cause ribosome stalling during translation may also be NGD targets.

The SKI (Superkiller) -exosome is a highly conserved 3'-5' exonuclease complex, which is essential for the proper functioning of normal mRNA degradation and different RNA quality control systems – such as NGD and NSD –, furthermore in its absence, the silencing system targets normal transcripts. A recent study proposed that in plants RST1 (Resurrection1) and RIPR (RST1 interacting protein) form a complex and that they also interact with the SKI and the exosome. They demonstrated that these plant-specific (RST1-RIPR) complex is cooperate with the SKI-exosome system to degrade silencing prone transcripts. Based on these findings, it has been hypothesized that RIPR and RST1 proteins may be involved in other SKI-exosome processes.

- The main purpose of my work was to verify each element of the eRF1 autoregulatory model at protein levels. In this part of the PhD program, we wanted to study the role of the NMD, an RNA quality control system in gene regulation.
- The other part of my work was to test, whether the position of the ribosome blocking polyadenine sequence within the ORF affects the efficiency of NGD degradation in plants.
- Moreover, I wanted to clarify whether uORFs that cause ribosome stalling may form another group of NGD cis-elements.
- Finally, I wanted to experimentally test whether RST1 and RIPR are also involved in the function of RNA quality control systems.

MATERIALS AND METHODS

Transgenic plants

To test predictions of the eRF1 autoregulatory model, eRF1 overexpressing *Nicotiana benthamiana* and *Arabidopsis thaliana* transgenic plants were generated. For agrobacterium-mediated transformation, *N. benthamiana* plants were grown in a growth chamber at 23°C under 16/8 light/dark and sterile conditions. Leaf disc transformation was carried out on 3 week old *N. benthamiana* plants. *A. thaliana* plants were grown in a growth chamber at 18/23°C (night/day) under long-day conditions. To generate transgenic *Arabidopsis* lines floral dip method were used on flowering plants. The integration and expression of the transgene was verified by PCR (Polymerase chain reaction) and western blot assays. Agroinfiltration-based transient gene expression assays were conducted with transgenic *N. benthamiana* plants. The eRF1 mRNA level was measured by quantitative RT-PCR (qPCR). Western blot was used to quantify the changes in transgenic protein expression.

Agroinfiltration and VIGS-agroinfiltration

Agroinfiltration was used to over- and co-express reporter genes. VIGS (virus induced gene silencing) was used for gene silencing in order to characterize the function of the gene of interest. For agroinfiltration and VIGS-agroinfiltration experiments, *N. benthamiana* plants were grown in the greenhouse. After agroinfiltration or VIGS treatment the plants were kept in a growth chamber at 23°C under 16/8 light/dark condition. For agroinfiltration 4-5 week old, for VIGS-agroinfiltration 3 week old plants were used. Leaves were agroinfiltrated with P14 silencing suppressor and/or reporter constructs. The *Agrobacterium* cultures, which are grown independently, are mixed, and the mixture is injected into the leaves of *N. benthamiana* plants (co-infiltration). Optical density of each bacterium culture are measured and diluted before infiltration (OD_{600} of each

culture was 0.4, or in the case of P14 OD_{600} was 0.2). Samples were collected 3 days after infiltration.

To initiate VIGS, two lower leaves of N. benthamiana plants were co-agroinfiltrated with three Agrobacterium cultures. One expressed P14, the second Tobacco Rattle Virus (TRV) RNA1 and the third expressed TRV RNA2 containing segments from N. benthamiana PDS (phytoene desaturase) gene or from PDS and a sequence from the target gene. PDS is used to monitor silencing. PDS silencing leads to photobleaching (leaf whitening), showing that PDS silencing was effective and suggesting that the silencing of the gene of interest is also effective. When the upper leaves are whitened (10-14 days after VIGS inoculation), leaves under the whitened ones were agroinfiltrated with Agrobacterium cultures expressing the RNA quality control test construct and the P14 control. Total RNA was extracted 3 days after agroinfiltration with the reporter construct. qPCR assays confirmed that the VIGS selectively and efficiently reduced the target mRNAs. RNA gel blot assays were carried out to monitor the changes in the mRNA expression of the reporter constructs.

Plasmid construction

Gene constructs used for the experiments were cloned into Bin61S binary plasmid or its derivatives and then each vector is introduced into a C58C1 *Agrobacterium tumefaciens* strain with triparental mating.

DNA extraction and PCR

Total DNA was extracted from cca.100mg leaf. Samples were homogenized in liquid nitrogen followed by extraction buffer treatment. After that, phenol-chloroform extraction method was used. PCR reaction was carried out with Dream Taq Green PCR Master Mix (2x, Thermo scientific).

RNA extraction and Northern blot

Total RNA was extracted from cca.100mg leaf. Samples were homogenized in liquid nitrogen followed by extraction buffer treatment. After that, phenol-chloroform extraction method was used. Then 3 to 6 µg from the total RNA samples were separated on 1,5% denaturing agarose gel, then blotted using capillary technic and hybridized with radioactively labelled DNA probes. The radioactive signals were detected in a phosphoimager.

qRT-PCR

cDNA was synthesized with RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific) from DNAse I treated total RNA samples. qRT-PCR assays were carried out with Fast Start Essential DNA Green Master Mix (Roche) in a Light Cycler 96 (Roche) Real-Time PCR machine. Ubiquitin was used as an internal control for qRT -PCR assays. Paired t-tests were used to calculate the significance of the differences.

Protein extraction and western blot

Total protein was extracted from cca.100mg leaf. Samples were homogenized in liquid nitrogen followed by extraction buffer treatment. After that, the extraction was carried out with 2x Laemli solution. The total protein samples were separated on 10% polyacrylamide gel, then transferred to nitrocellulose membrane and probed with anti-HA, anti-Actin and anti-mouse IgG monoclonal antibodies. To quantify protein expression, the detected HA chemiluminescent signs were normalized to actin internal control (HA/Akt).

Enzyme activity measurement

Samples were collected from cca.100mg leaf. Samples were homogenized in lysis buffer, then the substrate of NAN and GUS enzyme were added to the lysate. The fluorescence was detected with Hidex Plate Chameleon machine.

RESULTS

Testing the autoregulation model of eRF1-1 in transgenic plants

Based on previous results, our group suggested that in plants the eRF1 level can be autoregulated, and this self-regulation is based on the eRF1 sensitivity of the specific structure of eRF1-1 mRNA. Based on the findings, if: 1) the NMD reduces the eRF1-1 mRNA level, 2) the RT can partially protect the eRF1-1 transcript from the eRF1-1 3'UTR induced NMD, and 3) high eRF1 protein levels inhibit readthrough, they hypothesized that in plants, the special 3'UTR structure of eRF1-1 allows negative autoregulation of eRF1. According to the model, increase in eRF1 protein levels can inhibit readthrough, thereby termination at the RT stop will be more frequent. Consequently, NMD can degrade 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. Our group previously verified each element of this model at mRNA level. During this part of my PhD program I wanted to test the main predictions of this model at protein level. For this, *N. benthamiana* and *A. thaliana* transgenic plants were generated with HA-tagged eRF1-1 fusion constructs. As the plasmid constructs had all of the regulatory elements of the eRF1-1 mRNA, we supposed that changes in the transgene expression could mimic the changes of the endogenous eRF1-1 expression.

Most of the HA-eRF1-1-st-T *N. benthamiana* t_0 and *A. thaliana* t_1 transgenic lines, which expressed the eRF1-1 coding and a RT-NMD 3'UTR region, expressed the transgenic protein to detectable levels. In these lines transgenic mRNAs were accumulated to high levels and efficiently spliced. In these eRF1 overexpressing plants, the endogenous NbeRF1-1 mRNA expression was reduced. We conducted transient gene expression experiments on the transgenic *N. benthamiana* plants to monitor the changes of the HA-eRF1-1 protein concentration. Increased eRF1 protein level could reduce the RT frequency and the endogenous NbeRF1-1 mRNA, the transgenic HA eRF1-1

mRNA and HA-eRF1 protein expression in transgenic *N. benthamiana* plants. NMD inhibition led to increased endogenous NbeRF1-1 mRNA expression and elevated transgenic HA eRF1-1 mRNA, which was followed by an increase in HA-eRF1 protein level. In absence of NMD, further increase of eRF1 protein level (eRF1-3 overexpression) failed to decrease the endogenous NbeRF1-1 mRNA, the transgenic HA eRF1-1 mRNA and protein expression. This observation confirmed that the decrease in eRF1 mRNA and protein levels was caused by NMD degradation. Thus, results obtained from transgenic *N.benthamiana* plants strongly support the eRF1 autoregulatory model. However, in those plants where the eRF1 level was extremely high or low, the eRF1 autoregulatory circuit didn't operate properly.

In plants, long A-stretch is a position-dependent NGD cis-element

Previously our group have shown that a long A-stretch in the coding region induces NGD mediated mRNA cleavage in plants. Recent results suggest that in yeast, the position of the stall sequence is crucial for NGD activation. Translation blocking sequences induce NGD-mediated cleavage efficiently only if they are present at least ~100 nt from the start codon. It was demonstrated that ribosome collision is required for NGD activation. In line with this, the farther the stall sequence from the start codon, the more ribosomes can collide with each other and the more efficient the mRNA cleavage and degradation.

To test whether plant NGD is also sensitive to the position of the stall sequence, we altered the position of the 36A-stretch in the coding region and then assessed the intensity of NGD. We demonstrated that in plants, like in yeast, the NGD induction occurred only if the stall sequence is present at least ~100 nt from the start codon. Moreover the longer the distance from the start codon, the more efficient the NGD. We speculate that ribosome collision is also a prerequisite for NGD activation in plants.

Most of the studied uORFs that cause ribosome arrest, are not subjected to NMD and NGD degradation

Among uORFs, there are conserved peptide sequence encoding uORFs (CPuORF), and minimum uORFs (minuORF). Some of these are known to cause ribosome arrest during translation. Generally, mRNAs with stalled ribosomes are identified and degraded by RNA quality control systems. To test whether the NMD or NGD play a role in the degradation of these ribosome arrest uORFs, we chose five transcripts encoding sequence-dependent arrest uORFs, and monitored the NMD and NGD sensitivity of these mRNAs.

Only one construct, which contains a 50 aminoacid long CPuORF showed elevated mRNA level in the absence of NMD, while the other tested uORFs have failed to trigger NMD. Transcript cleavage is a key step during NGD, then the 3' and 5'cleavage fragments are degraded by the XRN4 and the SKI-exosome. Among the tested uORFs, only the minuORF induced mRNA cleavage, and the 3' cleavage product accumulated in the absence of XRN4. We have demonstrated that the 5' cleavage product is eliminated by the SKI-exosome, however the HBS1 and Pelota, the key factors of NGD are not involved in the decay of the 5' fragment. Thus, the NGD machinery does not play a role in the degradation of these cleavage products.

Identification of NGD and NSD trans-factors in *N. benthamiana*: the role of RIPR and RST1 proteins

It was proposed that the *Arabidopsis* RST1 and RIPR proteins connect the SKI complex to the exosome. Both proteins are conserved in flowering plants, thus we hypothesized that they perform a similar function in *N. benthamiana*. A Recent study revealed that the siRNAs from silencing prone transcripts have accumulated in *A. thaliana ripr* and *rst1* mutants similar to SKI-exosome mutants. Previously it has shown that SKI-exosome is required for the degradation of NSD target transcripts and for the elimination of the 5' cleavage fragments

generated by NGD, minuORF, miRNA or viral siRNA programmed RISC. We would like to clarify, whether RST1 and RIPR are also required for these SKI-exosome functions.

To test this assumption, degradation of NSD, NGD, miRNA, siRNA and minuORF reporter transcripts was studied in RST and RIPR silenced *N. benthamiana* plants. Our results indicate that the RST1-RIPR complex is required for all tested SKI-exosome activities.

CONCLUSIONS AND RECOMMENDATIONS

The role of plant eRF1 autoregulation

To maintain translation efficiency, the expression of the eRF1 key translation termination factor has to be strictly controlled. Inadequate eRF1 protein level may be harmful to both eukaryotes and prokaryotes.

Our data suggest that eRF1 autoregulation plays an important role in maintaining proper eRF1 protein level, thus it is essential for efficient translation and normal development. Our data suggests that in plants, the intensity of RT, NMD and normal translation termination depend on eRF1 protein concentration. As the proper function of all three types of termination coupled events are essential, precise regulation of eRF1 levels is crucial. As the eRF1-1 autoregulatory circuit might buffer fluctuations in eRF1 protein level, NMD and RT, we propose that this complex regulatory system helps to keep the balance between efficient translation termination, RT and NMD.

The eRF1-1 RT-NMD 3'UTR autoregulatory mechanism is conserved among flowering plants and it has an important role in the precise regulation of eRF1 protein level. However in yeasts and plants, both over- and underexpression of eRF1 lead to altered phenotype, the *eRF1-1* mutant *Arabidopsis* plants have not shown altered phenotype under laboratory conditions. We hypothesize that the fine-tuning of eRF1 expression may play an important role under stress

conditions. Therefore, it would be interesting to examine the development of eRF1-1 mutants under different stress conditions.

Long A-stretches are plant position-dependent NGD cis-elements

We demonstrated that in plants – like in yeast – the efficiency of NGD cleavage also depends on the position of the stall sequence. The longer the distance from the start codon, the more efficient the NGD activation. We speculate that ribosome collision is also a prerequisite for NGD activation in plants. We hypothesize that although collision of two or three ribosomes (which can occur if the stall sequence is located ~100 nt from the start codon) might be enough for weak NGD activation, collision of more ribosomes induces plant NGD more efficiently. This gradual regulation may be important, because in plants slowing down translation and sort-term ribosome stalling are frequent on normal transcripts. We hypothesize that NGD induction occurs only when the ribosome permanently blocked, while short-term ribosome stalling during normal translation do not activate NGD.

In plants, CPuORF and minuORF are not NGD cis-elements

Only a few sequence-dependent arrest uORFs have been reported in plants to date. Because ribosome stalling in the coding region could activate NGD system, these uORFs could form another group of NGD cis-elements. The key step of NGD decay mechanism is mRNA cleavage. However, based on our results, the tested CPuORFs did not induce mRNA cleavage. A possible explanation for this is that the majority of the tested CPuORFs are shorter than 100 nt. Based on our previous results, translation blocking sequences induce NGD-mediated cleavage efficiently only if they are present at least ~100 nt from the start codon. On a CPuORF longer than 100 nt, the ribosome stalls at the stop codon and it probably leads to inefficient termination and it may cause NMD degradation of the transcript. Endonucleotic cleavage always leads to rapid and efficient degradation of the mRNA. However, it is conceivable that the studied

CPuORFs may have a fine-tuning role, so fast mRNA degradation immediately after ribosome stalling is undesirable.

A recent study revealed that the minuORF induced mRNA cleavage. We have demonstrated that the 5' cleavage product of a minimum ORF containing mRNA is degraded by the SKI-exosome however, the Pelota and HBS1 NGD trans-factors are not required for its decay. Thus the NGD is not involved in the degradation of a minuORF containing mRNA. The proposed main role of Pelota-HBS1 complex is to remove the 80S ribosome from the 3'end of the 5'cleavage products of NGD, thereby allowing the SKI-exosome system to degrade these fragments. The MinuORF induced cleavage occurs 13-14 nt upstream of the P-site in the 5' UTR. Thus it is likely that a scanning ribosome (only a small subunit) runs to the 3' end of the 5' fragment. We propose that the Pelota-HBS1 complex is not required to remove the scanning ribosome from the 3' end of the 5' MinuORF cleavage fragments but it is essential to disassemble the 80S ribosome from the 3' end of the 5' cleavage products generated by NGD. However, as silencing is never complete, we formally cannot exclude that the reduced amount of Pelota-HBS1 complex in the VIGS plants is sufficient to remove the scanning ribosome but not the 80s ribosome from the 3' end of the different 5' cleavage fragments.

The putative role of plant RST1 and RIPR proteins

We found that RST1 and RIPR are involved in all tested cytoplasmic SKI-exosome activities. Thus these proteins are required for the SKI-exosome mediated degradation of NSD target transcripts and for the elimination of the 5' cleavage fragments generated by NGD, minuORF, miRNA or viral siRNA programmed RISC.

In mammals, the SKI-exosome mainly involved in the function of the RNA quality control systems, while normal RNA degradation occurs mainly through the XRN1 decay pathway. In contrast, in plants, the SKI-exosome complex is also involved in normal RNA degradation processes. We investigated

the role of RIPR and RST1 protein in RNA quality control, however, further experiments are needed to determine whether they are also involved in the function of general RNA degradation pathways.

Immunoprecipitation-MS assays revealed that RST1 is associated with RIPR, SKI7 and the exosome, while RIPR mainly purifies with the SKI complex in addition to RST1 and SKI7. In yeast and mammals, SKI7 links the SKI and the exosome complexes and it is essential for the SKI-exosome activities. In plants SKI7 also links the SKI and exosome complexes. However, it is likely that in plants, SKI7 is not essential for SKI-exosome mediated decay.

Based on these findings, it is conceivable that in flowering plants, RIPR and RST1 proteins are able to link the SKI and exosome complex even in the absence of SKI7, thus they promote RNA surveillance activity of the SKI-exosome system.

NEW SCIENTIFIC RESULTS

1. We have generated eRF1 overexpressing *N. benthamiana* and *A. thaliana* transgenic lines, which have proved to be suitable for studying the eRF1 autoregulatory mechanism at the protein level.

2. We have verified each element of the proposed eRF1 autoregulation model at protein levels. We have demonstrated that the elevated eRF1 protein level reduced the eRF1 expression by reducing translational readthrough at the RT stop codon of eRF1-1 mRNA, thereby the NMD could degrade the transcript efficiently and it led to decreased eRF1-1 mRNA and protein levels.

3. We have proven that in plants, the long A-stretch is a position-dependent NGD cis-element: the longer the distance of the A-stretch from the start codon, the more efficient the NGD induced cleavage of the transcript.

4. We have demonstrated that the minuORF is not an NGD cis-element: the 5’ cleavage product of a minimum ORF containing mRNA is degraded by the

SKI-exosome however, the Pelota and HBS1 NGD trans-factors are not required for its decay.

5. We have demonstrated that the *N. benthamiana* RST1 and RIPR proteins are involved in the decay of NSD target transcripts and in the elimination of the 5'cleavage fragments generated by NGD, minuORF, miRNA or viral siRNA programmed RISC.

PUBLICATIONS

Publications in international journals:

AUTH, M., NYIKÓ, T., AUBER, A., & SILHAVY, D. (2021) The role of RST1 and RIPRproteins in plant RNA quality control systems. *Plant molecular biology, 106*(3), 271–284.

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