Connections between RNA- and protein-based quality control systems in plants

Closing report

Quality control systems are essential in eukaryotic cells, these systems **ensure the fidelity of gene expression.** RNA quality control systems identify and eliminate aberrant transcripts thereby preventing generation of potentially detrimental faulty proteins. Protein quality control systems correct or degrade aberrant protein product. The critical question in quality control, how these systems can distinguish between the correct and aberrant products. It is very challenging as any sequentially and structurally completely different RNAs and proteins could be aberrant in many different ways and faulty products are present at very low concentration relative to the normal gene products. Recent results have shown that translation is a critical point at which many quality control mechanisms identify the aberrant products. Although quality control systems are intensively studied in animals and yeast, **plant quality controls have been barely studied.** During this research program (K 136513, titled "Connections between RNA- and protein-based quality control systems in plants") **we have analyzed the operation and connection of RNA and protein quality control systems in plants.**

Four post-transcriptional RNA quality control systems function in plants, the RNA Silencing, the Nonsense-Mediated mRNA Decay (NMD), the No-Go Decay (NGD) and the Non-Stop Decay (NSD) systems. The plant silencing system is well characterized, it is the most important plant antiviral system and it plays a critical role in quantitative gene expression (in addition to functioning as quality control system). The three other RNA quality control mechanisms are barely analyzed in plants. Our group previously described and analyzed the plant NMD system (Kerényi et al., 2008; Nyikó et al., 2013) and identified first the NGD and NSD in plants (these results were presented as preliminary results in the project proposal) (Szádeczky-Kardoss, et al., 2018; Szádeczky-Kardoss, et al., 2018). During this program **we** have **studied NMD, NSD and NGD RNA quality control systems and their links with other RNA** quality controls **and** with RQC (see below) **protein quality control system in plants.** The **NMD, NGD and NSD are translation coupled cytoplasmic quality control systems**, while silencing is not coupled to translation and acts in both nucleus and cytoplasm. Silencing degrades double-stranded and cap/or polyA less aberrant transcripts and inactivates nucleic acids showing strong homology to these aberrant gene products (act in both *cis* and *trans*). The translation coupled quality control systems function only in *cis*, they identify the faulty mRNAs during translation and then eliminate them but do not target the homologous sequences for inactivation. **NMD** recognizes and **degrades** aberrant **mRNAs having premature termination codons, NSD** decays **stop codon-less faulty mRNAs, while NGD** eliminates imperfect **mRNAs containing a translation elongation blocking segment.**

The mechanism and connections of plant NGD

Cis elements of plant NGD

NGD identifies aberrant mRNAs having elongation inhibiting sequences and cleaves these transcripts. We **identified first NGD** (Szádeczky-Kardoss, et al., 2018) **in plants** (it was preliminary results in the application and published before the program effectively started) by

studying the expression reporter transcripts having different elongation slowing-inhibiting sequences, which activated NGD in yeast (stem-loops, rare codons, prolins, poly-basic amino acids and poly adenine sequences etc.). We have shown that in plants only the **long A-stretches** (poly adenine sequences) (Szádeczky-Kardoss, et al., 2018) **and the very strong stem-loop (SL) structures** (Auth et al., 2021) **activates NGD.** We found that both the strong A-stretch and SL structure trigger NGD machinery, which cleaves the mRNA, thereby preventing further translation from the faulty transcript. We demonstrated that **A-stretch is a length and position dependent NGD *cis* elements,** 72 consecutive A induced very efficient cleavage, while 36A and 24A resulted in cutting 20-50% and 2-10% of the transcripts (Szádeczky-Kardoss, et al., 2018). As such long A-sequences are not present in plant transcripts, we propose that NGD mainly targets prematurely polyadenylated transcripts. More interestingly, the NGD cleavage rate is strongly depended on the position, A-stretches closer than 100nt to the start codon did not trigger NGD-mediated cleavage, longer distance from the start leads to gradually more efficient NGD (Auth et al., 2021). It is not known how aberrant transcripts having NGD activating features close to the start codon are inactivated in plants. We also found that only strong SL structures can activate NGD (Lakatos unpublished). As strong SL structures are rare in plant transcripts but frequently present in viral and transposon RNAs, SL-induced NGD could play antiviral and transposon inactivating role in plants. In yeast, two models were proposed how NGD functions. In both models blocked translation leads to ribosome collision that activates NGD. One model proposes that colliding ribosomes recruit CUE2 SRM-domain containing protein (D'Orazio et al., 2019), which cleaves the mRNA in the second ribosome, while alternative model proposes that collision of at least three ribosomes is required for NGD and that cutting occurs more upstream between ribosomes (Navickas et al., 2020). To define whether the NGD cleavage occurs close or more upstream from the stalling sequence in plants, we **mapped the 3'ends of 5' NGD cleavage products** of reporter transcripts containing an A-stretch (36A) or a strong SL. **Degradation of the cleavage product was inhibited by co-expressing Pel2**, an *Arabidopsis* protein that inhibits the degradation of NGD 5' cleavage fragments (Szádeczky-Kardoss, et al., 2018). We identified several **3'ends**, all of them **were upstream close to the stalling region** (independently whether the stalling was caused by of an A-stretch or an SL structure (Lakatos unpublished). Thus we concluded that **NGD cleavage in plants occur upstream very close of the stalling sequence, which is consistent with the CUE2-like second ribosome cleavage model.** Unfortunately, plant ortholog of CUE2 could not be identified, so the plant NGD-ase is still not known.

Trans factors of plant NGD

We previously identified couple of key components of plant NGD (except the NGD-ase): the eRF1 and eRF3 translation termination factor paralog Pelota and HBS1 proteins, and Ski2 and XRN4 factors, which are directly involved in the degradation of NGD cleavage product. We proposed that **Pelota-HBS1 complex removes the ribosome from the 3' end of 5' NGD cleavage fragment and that it is required for the decay of this fragment.** Then the **5' cleavage fragment is degraded by the SKI-exosome** complex (Szádeczky-Kardoss, et al., 2018). In this program **we have shown** that in plants during the elimination of NGD cleavage fragment the **SKI and exosome** complex are **connected by the RTS1-RITS** complex, while in yeast and animals SKI7 connect the SKI-and exosome complexes (Auth et al., 2021). The **3' NGD cleavage fragment is eliminated by XRN4** (Szádeczky-Kardoss, et al., 2018).

NGD and NSD are strictly linked in plants and NGD regulates silencing amplification

We previously described that **NGD and NSD are more strongly connected in plants than in yeast** (Szádeczky-Kardoss, et al., 2018). NSD targets two types of stop codon-less transcripts, (1) premature transcription termination-polyadenylation generated aberrant mRNAs having polyA but not stop codon and (2) endonucleolytic cleavage generated faulty transcripts lacking both stop codon and polyA. If polyadenylation-cleavage occurs in the coding region, the generated transcript will have polyA tail but lack the stop codon, thus the translating ribosome runs into the long polyA, which induces NGD cleavage and degradation. If **an endonucleolytic cleavage occurs in the coding region of a translated mRNA** (what is frequent and important, plant miRNAs cleave target transcript in the coding region), the 3' cleavage fragment is degraded by XRN4, while **the 5' cleavage product is eliminated by the Pelota-HBS1-SKI-exosome pathway** (Szádeczky-Kardoss, et al., 2018; Szádeczky-Kardoss, et al., 2018). As RNA silencing amplification generates deleterious secondary siRNAs from the miRNA cleavage fragments if they are not degraded quickly, we proposed that these **connections between miRNA-mediated silencing** cleavage **and NSD-mediated rapid decay** of cleavage fragments **are important in plants.** We started to generate different complex mutants to prove that NSD plays important role in controlling silencing amplification (as it was suggested in the original work plan) but before we could establish the multiple mutants, another group conducted these assays. They confirmed our model, indeed NGD plays a role in silencing regulation in plants (Vigh et al., 2022). Thus we focused on connections between NGD and NMD and NGD and RQC (see below). We found that **NMD and NGD are not linked,** NGD and NSD operated efficiently in the absence of NMD, and NMD functions in the lack of NGD, NSD components. **In contrast, we found that NGD and RQC are connected.**

RQC degrades protein products of stalled ribosomes

RQC (Ribosome associated Quality Control) is a conserved protein quality control systems, which degrades nascent proteins present on the stalling ribosomes. If an mRNA contains structures that inhibit elongations, quality control systems have to solve 3 issues: degrade the truncated proteins present on the stalled ribosome, decay the mRNA to prevent further truncated protein generation and rescue-recycle the ribosomes. Ribosome stalling activates NGD, which degrades the aberrant transcript, while RQC eliminates the potentially detrimental nascent protein. It is hypothesized that both NGD and RQC facilitate ribosome recycling. RQC has not been described in plants. During this project, we have **established an efficient reporter system to study plant RQC, identified the RQC *cis* elements and two *trans* factors** and studied the connections between RQC and RNA quality control systems (Lakatos unpublished).

HA-RED-GFP reporter construct was generated, and then different potential ribosome stalling and potentially RQC inducing elements were inserted in frame between RED and GFP. These reporters were expressed in transgenic plants, and then protein expression was studied. We have found that when **36A** was inserted **between RED and GFP, in addition to the full length protein a shorter protein accumulated to detectable levels**. By inserting a FLAG tag between (1) the RED and 36A or (2) between the 36A and GFP, we could map that the shorter protein is the nascent protein that was present in the ribosome stalling at the 36A region (Lakatos). In yeast, ribosome stalling and nascent protein accumulation can be induced by 10(AAA) codons and by 7AAG3AAAcodons, while the same lysine encoding 10(AAG) does not lead to nascent protein generation. Thus it was proposed that two signals are required for RQC induction (double signal RQC model), a polylysine stretch that slows down

translation and an A-stretch, which can adopt conformation that inhibits binding to the A-site. We expected that model is also valid for plants. However we found that **this double signal RQC model cannot be verified in plants**, 12AAG and 9AAG3AAA failed to induce RQC, while 12AAA led to nascent protein accumulation. Next we wanted to identify the components of plant RQC. We have transiently inactivated the potential tobacco orthologs of the yeast RQC systems, and then HA-RED-36AAA-GFP reporter was expressed in these plants. We found that **in two mutants,** one that showed moderate similarity to the RQC2 and one to the Ltn1 components of the yeast RQC system, the nascent/full-length protein ratio was dramatically increased suggesting that the nascent protein degrading **RQC was impaired**. Thus we concluded that ribosome stalling leads to rapid RQC mediated degradation of the nascent protein and that, **plant orthologs of RQC2 and Ltn1 are essential for RQC** mediated nascent protein decay**.** We have also studied how RQC and RNA quality control systems are linked. We found that **NMD or NGD** components **are not required for RQC and RQC components are not required for RNA quality control** (Lakatos unpublished)**.**

Importantly, **NGD and RQC operates on the same target** transcripts, we found that HA-RED-36AAA-GFP transcript is NGD cleaved, although the cutting is inefficient (10-20%). In worm (but not in yeast), NGD factors facilitate RQC. In plants, it does not happen, NGD inactivation did not modify RQC efficiency. So in plants, NGD and RQC are linked, they **both inhibit the accumulation of truncated nascent proteins from transcripts having stalled ribosome,** NGD by cutting the transcript, while RQC by degrading the nascent protein (Lakatos unpublished). We propose that RQC plays an important role in stress responses, during stress condition ribosome stalling is frequent (tRNA shortage, non-proper folding), moreover it could be essential at certain developmental phases. Indeed, we found that **RQC2 homozygous mutant is not viable, it is embryo defective.** Further studies are required to clarify whether RQC is essential or RQC2 protein has additional function. The Ltn1 hypomorph mutant is viable (Péter unpublished). We have been testing the stress tolerance of RQC hypomorph mutants. The manuscript, in which we will first describe RQC in plants will be submitted soon (Lakatos unpublished).

Operation of NMD regulates translation termination in Angiosperms and different fungi

NMD regulates translation termination in Angiosperms and different fungi

NMD is a conserved eukaryotic translation termination coupled quality control system that identifies and degrades premature termination codon containing aberrant transcripts. We previously described NMD in plants, we recognized the *cis* and *trans* factors and the regulation of plant NMD (Kerényi et al., 2008; Nyikó et al., 2013). We also demonstrated that **in plants NMD is involved in the complex autoregulatory circuit that controls the expression of the eRF1 key translation termination factor** that binds to the stop codon, release the peptide and plays a role in ribosome recycling (Nyikó et al., 2017)**.** During this project **we studied the evolution of this very specific NMD controlled eRF1 autoregulatory mechanism.**

eRF1 is always present in multiple copies in plants and one of them (eRF1-1) has a unique 3'UTR structure (called readthrough-NMD structure), which is critical for eRF1 regulation. Th eRF1-1 stop codon is present in a readthrough promoting context, while the 3'UTR contains NMD inducing elements (Nyikó et al., 2017). We found that similar readthrough-NMD 3'UTR structure can be recognized in the eRF1 mRNAs of different fungi including Neuropora and Aspergillus (but not in other eukaryotes). We have shown that **similar eRF1**

autoregulatory circuits, in which NMD plays a key role, have evolved independently in plants and fungi (Kurilla et al., 2020)**.** However, this regulatory circuit is absolutely conserved in all angiosperms, while in fungi it was lost in several branches. The eRF1 autoregulatory circuit: if eRF1 protein level is high, termination occurs at the stop codon of the eRF1 transcript having readthrough-NMD 3'UTR structure, thus eRF1 protein is synthesized. However, it also activates NMD, which degrades the eRF1 transcript, thereby reducing eRF1 direct translation efficiency (protein synthesized from a single normally spliced and exorted transcript). In contrast, when eRF1 protein level is low, readhtrough occur frequently on the eRF1 mRNA (having readthrough-NMD structure), which stabilizes the mRNA by removing the NMD inducing proteins from the 3'UTR. Then, from the stabilized transcript several eRF1 proteins can be generated. We showed that **this eRF1 regulatory system also controls the efficiency of NMD,** high eRF1 protein level enhances NMD activity (Kurilla et al., 2020; Nyikó et al., 2017). Thus intense NMD leads to reduced eRF1 protein level, which results in decreased NMD efficiency.Taken together, we unravelled that such a sophisticated eRF1 autoregulatory system that can also control NMD intensity has evolved in different eukarytotic banches. Interesting, the key translation termination factor (eRF2) is also controlled by an autoregulatory circuit in bacteria, however that one is mechanistically (and evolutionary) different.

Plant NMD degrades target transcripts exonucleolytically

In animals, NMD initiates aberrant transcript degradation by inducing SMG1-mediated transcript cleavage. **Previously we showed** that SMG1 is not present in Arabidopsis and that **plant NMD triggers exonucleolytic degradation** of faulty mRNAs. During the project it was **reported the eRF1-1 is degraded by NMD mediated endonucleolytic cleavage in plants** (Nagarajan et al., 2019). It was an unexpected but very important and inspiring result, thus **we performed several assays to clarify the details of it.** We **failed to detect NMD cleavage product** in Arabidopsis and tobacco. It is possible that plant NMD has different degradation pathways and it depends on growth condition, however, the more likely explanation is that an **artefact** was detected as NMD cleavage product and plant NMD does not cut target transcripts. Moreover, in collaboration with the Kufel group, we have identified a new plant NMD component, which facilitates decapping (Sulkowska et al., 2020). It further supports our previous findings that in plants, NMD initiates rapid deadenylation-decapping followed by exonucleolytic degradation.

Does NMD function in Chlamydomonas haploid green alga?

To better understand the evolution of plant NMD, we studied NMD in *Chlamydomonas reinhardtii* haploid green alga. NMD was analyzed in many eukaryotes but all of them were diploid (or polyploid). NMD is obviously advantageous in a diploid, if the mutant is heterozygous and one allele generates NMD target premature termination codon containing transcripts, NMD selectively degrades the faulty transcripts but it does not modify the expression of the normal mRNAs transcribed from the correct allele. However, in a haploid if only prematurely terminated transcripts are generated from a mutant gene, it is not clear which one is better, to degrade them or let them to translate into truncated proteins. We found that plant NMD *cis* elements, the long 3'UTR and intron in the 3'UTR also lead to low expression in Chlamydomonas. We hypothesized that NMD targets these transcripts in Chlamydomonas, as well as in plants. UPF1 is the key NMD factor in all eukaryotes. We identified a Chlamydomonas in which the UPF1 was mutant and then we compared the expression of the reporters in wild-type and *upf1* mutant. Surprisingly, we found that long 3' UTR and intronic 3'UTR containing transcripts are also downregulated in *upf1* mutant.

Moreover, we conducted comparative RNA-seq assays and found that the transcriptome is barely altered in the *upf1* mutant. Thus we concluded that **Chlamydomanas,** like all other Eukarytoes analyzed so far, **downregulates mRNAs having premature termination codon, but it inactivates them by an NMD independent mechanism.** We also found that high C/G ration in the 3'UTR also leads to low express in the the green algae, which might be important to quality control (Silhavy unpublished). The manuscript in which describe that the Chlamydomans "NMD" system operates differently will be submitted soon.

NMD and TFIIS transcriptional RNA quality control are both involved in heat-stress responses

We have also analyzed the role of quality control factors in biotic and abiotic stress responses. Unfortunately, other groups outcompeted us with the virus responses (Ge et al., 2023; May et al., 2018). Thus we focus our attention to abiotic responses. In collaboration withthe Csorba group, we study the role of quality control systems in heat-stress responses. We found that TFIIS transcription coupled RNA quality control system plays a role in heat-stress adaptation, in the absence of it, under high temperature the transcriptome is dramatically altered and the plants are more sensitive to high temperature. Moreover, splicing pattern is aberrant, several prematurely terminated mRNAs are expressed (Szádeczky-Kardoss et al., 2022). Therefore we tested the role of UPF1 in heat-stress response. We found that the *upf1* mutant is sensitive to high temperature and that the *upf1-tfiis* double mutant shows strong phenotype at both normal and elevated temperature (Szaker unpublished). These interesting data suggest that the two different RNA quality control systems act in collaboration to ensure fidelity of gene expression and to adapt to high temperature. We have generated other double mutants (*pelota-tfiiis, ltn1-tfiis*), however the stress assays have not been conducted.

RNA silencing plays moderate role in photomorphogenesis regulation

Quality control systems could be also involved in the regulation of normal gene expression (in addition to eliminating aberrant products), thus might play role in developmental controls. We have **studied the role of RNA silencing role in photomorphogenesis.** Moving the plants from dark to light (de-etiolation) induces dramatic gene expression and developmental responses. Thus we wanted to study the role of silencing system in this developmental shift. Surprisingly we found that total miRNAome and the AGO-coupled active miRNAone are only slightly modified during de-etiolation except the miRNA163, suggesting the **silencing plays moderate role in photomorphogenesis** (Lakatos et al., 2022)**.**

Planned publications

We want to submit at least three more papers strongly related to this program, (1) in which we describe that Chlamydomonas inactivate premature termination containing transcript in a UPF1-independent manner (referred to as Silhavy unpublished), (2) in which we will first describe RQC in plants and it connections with NGD (Lakatos unpublished) and (3) in which we will present the unexpected finding that the translation-termination coupled NMD and the transcriptional TFIIS quality control systems act cooperatively in heat stress response. Paper 1 and 2 will be submitted this year, while paper 3 will be published next year.

In summary: we think that program was successful; our results contributed significantly to our knowledge about the operation, connection and function of RNA- and protein quality control systems in plants.

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