Final Report

Quality control systems ensure that only perfect proteins can accumulate in the cells. In eukaryotes, **four RNA quality control systems, the RNA silencing, the nonsense-mediated decay (NMD), the no-go decay (NGD) and the nonstop decay (NSD) systems** have been described. These systems identify and degrade different aberrant transcripts. In addition to eliminating the faulty mRNAs, the RNA quality control systems frequently play an important role in the regulation of normal transcripts.

RNA silencing identifies and eliminates double-stranded transcripts (and silences the homologous nucleic acids) but it also degrades non-polyadenylated or non-capped aberrant mRNAs. The NMD system degrades aberrant transcripts having premature termination codons, while NGD decays mRNAs containing ribosome stopping structures in the coding region. NSD eliminates faulty mRNAs lacking the stop codon.

While the yeast and mammalian RNA quality control systems have been very intensively studied, much less is known about the mechanism and function of RNA quality control systems in plants. Indeed, **when this program started,** the **plant** RNA silencing system was well understood, but the **NMD system was barely characterized and the NGD and the NSD systems were not described in plants yet.**

The **major goal of this program was to better understand the operation and biological function of RNA quality control systems in plants**. To aim this, we wanted to clarify whether NSD and NGD systems function in plants. Moreover, we wanted to identify the most conserved NMD target plants genes (assuming that the most conserved targets are the biologically most important ones). During the program **we** have **characterized** the operation of **NSD and NGD systems** and unraveled that these systems are strongly connected **in plants**. Unexpectedly **we found that NSD and RNA silencing** quality control **systems function cooperatively in plants**. We have also **defined the NMD target transcript in tobacco and in the model moss and alga species** (*Physcomitrella patens* and *Chlamydomonas reinhardtii*). We have found only very few conserved NMD targets. However, our results **show that the NMD autoregulatory mechanisms are highly conserved** and that **NMD**, which is translation termination coupled quality control system, **regulates the expression** of the key translation termination factors (**eRF1**) in alga as well as in plants.

We believe that **our results significantly contributed to our understanding of the** mechanism and function of **plant RNA quality control systems**. Our results were published in leading journals and based on the results of the program, two PhD students fulfilled the publication requirement of the PhD and couple of undergraduate theses were defended.

Characterization of plant NSD system

In yeast and mammals, the NSD system degrades the aberrant mRNAs lacking an in frame stop codon. To unravel whether similar system function in plants, NSD reporter transcripts lacking a stop codon and control mRNAs were transiently expressed in plant leaves. As the NSD reporter mRNA was expressed to very low levels relative to the control we concluded that NSD system function in plants. NSD (like NGD and NMD) is a translation coupled quality control system. Therefore our finding that translation inhibition led to dramatically enhanced expression of plant NSD reporter transcript confirmed that the low reporter mRNA level was due to NSD (instead of transcriptional silencing).

We have also identified two critical NSD factors, Pelota (or Dom34) and HBS1. Transient silencing of either factor led to dramatically increased expression of the NSD reporter

transcript. Complementation assays confirmed that Pelota is the key NSD factor, its overexpression could complement the deficiency of both Pelota and HBS1, while overexpression of HBS1 could complement only the lack of HBS1.
Stop codon less transcripts can be generated by two different ways, 1, by early polyadenylation, which leads to the accumulation of mRNA having a polyA tail, (called non-stop transcript) and 2, by endonucleolytic cleavage of normal mRNAs. If the cleavage occurs in the coding region, the 5' cleavage fragment is a stop codon and polyA tail less transcripts (called stopless transcript). We have shown that plant NSD efficiently degrades both non-stop and stopless transcripts.
As in plants the miRNAs of the RNA silencing system cleaves the target transcripts in the coding region, we hypothesized that the 5' miRNA cleavage fragments are stopless transcript and that these fragments are degraded by the NSD system. Indeed, using various transient assays we demonstrated that the NSD system is required for the decay of 5' cleavage fragments of miRNAs (as well as siRNAs), if the cutting occurs in the coding region. At the beginning of the program, in collaboration with other groups, transcriptome and degradome sequencing were conducted on the model tobacco species (publication 1). Using these data we could confirm the results of our transient assays. We found that a subset of the endogenous miRNA 5' cleavage fragments overaccumulated significantly in tobacco in which Pelota or HBS1 was inactivated. Similar results were obtained when we analyzed the accumulation of 5' miRNA cleavage fragments in wild-type, *pelota* and *hbs1* mutant Arabidopsis plants. These data confirm that the NSD system plays an important role in the degradation of miRNA 5' cleavage fragments. However, as not all 5' cleavage fragment accumulated to enhanced levels in the NSD deficient plants, it is likely that other RNA decay systems are also involved in the elimination of the 5' cleavage fragments.
Importantly, our results indicate that NSD and silencing systems cooperate in plants (and we postulate that the two quality control systems also collaborate in other eukaryotes). As silencing is essential for development and it is the most important antiviral system in plants, and because quick elimination of silencing generated 5' cleavage fragments is essential in plants to keep the small RNA homeostasis, the unraveled cooperation between NSD and silencing system could be very important.
Interestingly, while miRNA 5' cleavage fragments overaccumulated similarly in *pelota* and *hbs1* mutants, only the pelota deficient Arabidopsis plants showed obvious phenotype (slow growing). Thus we concluded that NSD is not essential in plants and that Pelota has additional function.
Finally, as both silencing and NMD systems are involved in pathogen defense, we postulated that NSD (and NGD) system could also play a role in plant protection. Surprisingly, we have found that the *pelota* mutant is less susceptible to the *Turnip Crickle Virus* (TCV) than the wild-type (but perhps not to other +strand RNA viruses). Other groups have shown that Pelota-deficiency in tomato results in Begomovirus resistance and that *pelota* mutant rice plants have enhanced salicylic acid levels and thus show increased bacterium resistance.
This part of the project was run in strong in-house collaboration with the Plant Virology group. We have published our NSD results in the *Nucleic Acids Research* (publication 3), while the manuscript from the *pelota* and *hbs1* mutant studies will be submitted soon to the *Plant Molecular Biology* journal.

Characterization of plant NGD system

NGD is a translation coupled eukaryotic RNA quality control system which targets mRNAs containing an translation elongation inhibitory structure. In yeast, if the elongating ribosome is stalled by a strong inhibitory structure, the NGD system is induced, and then the mRNA is

cleaved by an unknown nuclease and the cleavage fragments are quickly degraded. Various elongation inhibitory structures (referred to as NGD *cis*-elements) can activate yeast NGD. It was reported that stable stem-loop structures, premature termination codons, rare codons, polybasic amino acid stretches (poly-lysine and poly-arginine tracks), A-stretches and chemically modified nucleotides induced NGD in yeast. It was also shown that the nucleotide composition can be important, A-stretches activated NGD especially efficiently. Indeed, (AAA)n encoded lysine tracks were more effective cis-elements than (AAG)n encoded tracks.
We wanted to study whether NGD system also functions in plants. The effect of different *cis*-elements that induced NGD in yeast was experimentally tested in the *Nicotiana benthamiana* model plant. Relevantly, unlike in yeast, only long A-stretches triggered NGD. These long A-stretches induce an endonucleolytic cleavage, and then the 5' fragments are decayed in a Pelota-, HBS1-dependent manner. We also show that the 3' cleavage fragments were degraded by the XRN4 5'-3'cytoplasmic exonuclease. We demonstrated that plant NGD acts gradually, longer A-stretches induce cleavage more efficiently. For instance, 24 consecutive A lead to infrequent cleavage, 36A resulted in the cutting of 50% of the mRNAs, while 72 consecutive a induced cleavage at extremely high frequency (above 90%). As long A stretches are extremely rare in the coding regions, we concluded that NGD does not target physiological transcripts. It is likely that NGD degrades mRNAs having an aberrant or modified nucleotide, if they stop translation. NGD might be also involved in the elimination of the stop codon less but polyA tail containing non-stop transcripts. When the ribosome runs into the polyA tail, the NGD system is activated and cleaves the transcript upstream of the A tail. Then the 5' cleavage fragment can be degraded by the NSD system.
We have also studied how NSD and NGD targets are degraded. We found that the SKI-exosome 3'-5' exonuclease plays a critical role in the decay of NSD and NGD targets. Previously similar results were obtained in yeasts and mammals. Taken together, our data suggest that the NSD and NGD systems are highly conserved in eukaryotes, thus they might be already active in the common ancestor of all extant eukaryotes.
We have published our NGD results in the *Plant Science* (publication 4).
As a continuation of NGD and NSD studies, in a next program, we want to further analyze the interaction between various RNA quality control systems and their connections to the protein quality control systems.

Identification of conserved NMD targets

The other major goal of this program was to identify the conserved targets of plant NMD system. To aim this, RNA-seq assays were conducted from NMD deficient tobacco, moss and Chlamydomonas lines, and then the overexpressed mRNA sets were compared. Previously the NMD targets of Arabidopsis plants have been identified by other groups. Surprisingly, only 4-6 conserved targets can be found between the two dicots, Arabidopsis and tobacco. Thus NMD targets are quickly changed. Interestingly, many conservative alternative splicing products are common targets. Importantly, the core NMD factors, SMG7 and Barentz are conserved targets, indicating that the NMD autoregulatory circles are conserved and important. Moreover, we found that SMG8 is NMD controlled in tobacco and moss (in Arabidopsis SMG8 is not present). More interestingly, the eRF1 key translation termination factor was NMD regulated in Arabidopsis, tobacco and green alga. As NMD is a translation termination coupled quality control system, this highly conserved connection between NMD and translation termination regulation could be biologically very important. Thus we started to study the role of NMD in eRF1 regulation. Our data indicated that a complex and highly special autoregulatory circuit controls the eRF1 expression in plants, and that NMD is a key element of this control system. Our data also suggest that eRF3, the other key termination factor, is not regulated by NMD in Arabidopsis, while in tobacco it is present in two copies,

and one of them is NMD controlled. It further confirm the intricate connection between NMD and translation termination regulation.
Our results indicate that NMD function very similarly in Chlamydomonas and plants, NMD efficiently degrades mRNAs having unusually long 3'UTR and transcript having an intron more than 50 nt downstream of the stop codon. It is surprising that NMD operates so efficiently in the model alga, as Chlamydomonas is a haploid organism. In diploids, the elimination of premature termination codon containing aberrant mRNA is logical, as the normal transcript from the wild-type allele can function. However, elimination of aberrant transcripts containing premature termination codon in a haploid leads to null phenotype. An additional interesting observation is that, in Chalmydomonas (but not in plants), the nucleotide composition of the 3'UTR is also important, high A, U and C ratio leads to very reduced transcript levels. The molecular mechanism of this nucleotide selection and its biological function is still not known.
We have published the eRF1 results in the *Nucleic Acids Research* (publication 2), while the revised manuscript about eRF3 has been submitted (after minor revision) to *Plant Molecular Biology Reporter* (publication 5). The manuscripts about the tobacco and moss NMD targets and the description of Chlamydomanas NMD system will be submitted soon.

We think that the program was successful, it reached the major aims and significantly contributed to our knowledge about the mechanism and function of different plant RNA quality control systems.

Publications related to the project

IF=Impact factor of the journal in the year of the publication
Scimago= ranking of the journal in the most relevant Scimago category or categories

1, Baksa I, Nagy T, Barta E, Havelda Z, Varallyay E, **SilhavyD**, Burgyan J, Szittya G,
Identification of Nicotiana benthamiana microRNAs and their targets using high throughput sequencing and degradome analysis. *BMC Genomics.* 2015 Dec 1;16:1025. doi: 10.1186/s12864-015-2209-6. (**IF. 3.867,** Scimago Genetics 48/327 Q1)

2, NyikóT*, Auber A*, Szabadkai L, Benkovics A, Auth M, Mérai Zs, Kerényi Z, Dinnyés A, Nagy F, **Silhavy D#.** Expression of the eRF1 translation termination factor is controlled by an autoregulatory circuit involving readthrough and nonsense-mediated decay in plants. *Nucleic Acids Research*. 2017 Apr 20;45(7):4174-4188. doi: 10.1093/nar/gkw1303. **(IF. 11.561,** Scimago Genetics 7/340 Q1, D1**)** (#corresponding author)

3, Szádeczky-Kardoss I*, Csorba T*, Auber A, Schamberger A, Nyikó T, Taller J, Orbán TI, Burgyán J, **Silhavy D#.** The nonstop decay and the RNA silencing systems operate cooperatively in plants. *Nucleic Acids Research* 2018 May 18;46(9):4632-4648. doi: 10.1093/nar/gky279. **(IF. 11.561,** Scimago Genetics 7/340 Q1, D1**)** (#corresponding author)

4, Szádeczky-Kardoss I*, Gál L*, Auber A, Taller J, **Silhavy D#.** The No-go decay system degrades plant mRNAs that contain a long A-stretch in the coding region. (2018) *Plant Science*. 2018. Oct;275:19-27. doi: 10.1016/j.plantsci.2018.07.008. Epub 2018 Jul 21. **(IF. 3.712,** Scimago Plant Science 33/444 Q1, D1; Genetics 87/340 Q2**)** (#corresponding author)

5, Auber A, Nyikó T, Mérai Zs, **Silhavy D#.** Characterization of eukaryotic Release Factor 3 (eRF3) translation termination factor in plants (2018) *Plant Molecular Biology Reporter* **(IF. 1.844,** Scimago Plant Science 93/444 Q1; Mol. Biol. 233/406 Q3**)** (#corresponding author). Manuscript is under minor revision, the revised version has been submitted.

Graduate and undergraduate theses related to the project

Based on publications related to this program, both participating researchers I. Szádeczky-Kardoss and A. Auber fulfilled the publication requirements of their PhD programs and obtained the absolutorium. Couple of Bsc and Msc students were involved in this program. The thesis of A. Dinnyes (BME Msc), A. Zakar (BME Msc), G. Iski (SZIE Msc) and L. Gal (SZIE Bsc) are all directly related to this project.