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Identification of *cis* and *trans* elements involving recognization and degradation of premature stop codon containing mRNAs in plants

Ph. D. thesis

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INTRODUCTION

Regulation of gene expression is a complex, multiplying level process, which ensure the correct quantity and quality, spatial and temporal expression of protein-coding genes for the cells. To ensure the fidelity of these processes, the eukaryotic cell has evolved several quality control mechanisms. Such evolutionarily conserved mechanisms are the nonsense-mediated mRNA decay (NMD) and RNA silencing. Both of the two mechanisms recognize and quickly degrade aberrant mRNAs although they distinguish the aberrant RNAs in a different way. RNA silencing is an inducible defense pathway to specifically target and inactivate invading nucleic acids like viruses, transposons and transgenes. RNA silencing can be induced by double stranded RNAs (dsRNAs) and finally it leads to the degradation of homologous RNAs (Baulcombe, 2004). Double stranded RNAs could be formed in different ways; for example as a replication intermediate products of plant single stranded RNA viruses or aberrant transcript products after transgene insertion. NMD rids the cell of aberrant mRNAs having premature translation termination codons (PTC) and so, arrests the translation of potencially harmful truncated proteins (Behm-Ansmant, 2007). Severe developmental defects or lethal phenotype show the essential role of NMD pathway in NMD mutants in all organisms. Although NMD mechanism was learned in yeast, *Drosophila, C. elegans* and mammals, very little was known about plant NMD. At least two different pathways exist to distinguish the normal and premature termination codons. In yeast and invertebrates PTC is defined by the lenght of the 3' untranslated region (UTR) of a mRNAs (Gatfield, 2003, Amrani, 2004). If the 3' UTR is too long, terminating ribosome cannot interact with poly-A binding protein and the termination of translation is inefficient. NMD core complex involving UPF1, UPF2 and UPF3 proteins is recruited to the mRNA that induces fast degradation of it. In summary, long 3' UTR acts as a *cis*-acting element in yeast, while *trans*-acting factors are the UPF proteins. In mammals, PTC definition based on the position of introns; introns in the 3' UTR serve as *cis* elements of mammalian NMD (Nagy, 1998). After intron splicing, a four-protein complex called exon-junction complex (EJC) is deposited at exon-exon boundaries (Le Hir, 2000). Within the open reading frame, EJC is removed by ribosomes during the first round of translation. When an intron is located downstream to the first in-frame termination codon, EJC remains bounded on the mRNAs and serves as a binding platform for NMD *trans*-acting proteins resulted in fast degradation of the mRNA (Chang, 2007). In the beginning of our project, very little was know about plant NMD. Neither *cis*-acting elements nor *trans*-acting proteins of plant NMD were studied before.

MAIN OBJECTIVES

Main aim of our group is to study the plant quality control mechanism that liable for recognization and elimination of mRNAs harbouring PTC. Since this mechanism was hardly examined before in plants, we had to set up new reporter system which eligible for analizing plant NMD *cis*-, and *trans*-acting elements. We have elaborated an agroinfiltration based transient NMD assay system. Forasmuch agroinfiltration, and agroinfiltrated NMD reporter genes strongly induces RNA silencing also, the two RNA quality control system had to be separated by selective inhibition of RNA silencing. To counteract RNA silencing, most plant viruses express RNA silencing suppressor proteins. My object was identification of a viral silencing suppressor which inhibits RNA silencing but does not effect on NMD, so it could be use as a tool for separating of the two RNA quality control mechanisms.

(1) My first object was identification of *Pothos latent virus* (PoLV) silencing suppressor. The PoLV open reading frame (ORF) 5-encoded 14-kDa protein (p14), like the *tombusvirus* ORF 5-encoded P19, increases the severity of viral symptoms (symptom determinant; Rubino, 1997). I analyzed the RNA silencing suppression mechanism of p14 protein.

(2) Our main aim was to identify the *cis*-acting elements of the plant NMD system. Our colleagues showed that long 3' UTRs act as NMD *cis* elements in plants, similar as in yeast. They have shown that this effect was size-dependent, mRNAs with longer 3' UTR were more effectively targeted by NMD than transcript with shorter 3' UTR. Introns are efficient NMD *cis* elements in mammals. We next wanted to clarify if introns located in the 3' UTR could act as NMD *cis* elements in plants. My object was to study the effect of intron position on mRNA stability.

(3) To better understand plant NMD, we would like to identify and characterize NMD *trans* factors with respect to their involvement in long 3' UTR- and intron-based NMD pathways. To achieve these aims, we combined VIGS, a well-documented gene silencing approach in plants, with an agroinfiltration-based transient NMD assay (VIGS-NMD system). Using this system, we examined the role of plant ortholog of UPF1, UPF2 and UPF3 in NMD. My further aim was to study plant EJC components (Y14 and MAGO) and SMG7 roles in plant NMD.

METHODS

- For agroinfiltration, we used *Agrobacterium tumefaciens* C58C1 strain. Reporter genes were cloned into pBIN 61S binary vector and than conjugated to *A. tumefaciens* by triparental mating. For agroinfiltration, *Agrobacterium* cultures were grown and resuspended. Bacterium suspension was infiltrated into *Nicotiana benthamiana* and *Nicotiana tabacum* leaves. Agroinfiltration assays were visualized by hand-hold UV lamp and analized by Northern blot.

- For virus infection, *in vitro* transcription was carried out using infectious cDNA clones of plant viruses as templates. Virus transcripts were rubbed into *N. benthamiana* leaves.

- For virus induced gene silencing (VIGS) we used *Tobacco rattle virus* (TRV; Ratcliff, 2001). TRV has a bipartite RNA genome, binary plasmids contain the cDNA of RNA1 or RNA2. cDNA of RNA2 could harbour different gene sequences of plant endogeneous genes. To trigger VIGS, leaves of 21-day-old *N. benthamiana* were coinfiltrated with a mixture of three *Agrobacterium* cultures. One expressed P14, the second expressed TRV RNA1 (BINTRA6 vector), whereas the third expressed TRV-P VIGS vector or its derivatives. Virus infection can be visualized 3-4 day post-infiltration, while recovery (and silenced) phenotype evolves 10-12 day post-infiltration. In the recovered leaves endogeneous gene, which showes homology with the RNA2, expression decrease.

- mRNA levels were analized by Northern blot hybridization. RNA samples were collected 3 day after agroinfiltartion. For sRNA detection, low-molecular weight RNA gel blot was carried out (Silhavy, 2002). In that method, sRNA samples were denaturated and separated by 15% poliacrilamid gel.

- For analysis of RNA-protein interaction, we used gel mobility shift assay (EMSA). Total protein extracts were collected from agroinfiltrated or virus infected leaves. T4 PNK labeled synthetic sRNAs or [γ-32P]UTP-labeled *in vitro* RNA transcripts were used as long RNA probes (Silhavy, 2002). Protein-RNA binding reactions were separated on native polyacrilamid gel which was than vacuum-dried and scanned with Storm 840 Scanner.

RESULTS

1. We have demonstrated that *Pothos latent virus* (PoLV) ORF5 encoded 14 kDa protein (p14) is a RNA silencing suppressor. p14 inhibits transgene mRNA degradation and transgene specific sRNA accumulation in agroinfiltration assay. p14 strongly inhibits RNA silencing, however it does not have effect on NMD and in this manner, p14 can be used as a tool for separating of the two RNA quality control mechanisms.

2. We have shown that p14 is a double stranded RNA binding protein without any size selection, it can bind approximately the same molar excess of longer dsRNAs and shorter sRNA. P14, however, was not able to inhibit degradation of dsRNA but delayed the sRNA accumulation either in agrionfiltration assay or during viral infection. Infection of *Nicotiana benthamiana* plants with PoLVΔ14, a mutant PoLV that fails to express P14 leads to recovery phenotypes, while infection of PoLV leads to necrotic phenotypes. It strongly suggests that p14 suppressor effect on sRNA formation is essential for effective virus infection.

3. We have set up an agroinfiltration-based transient NMD test system. Taking the advantage of RNA silencing suppressor p14 we have shown that PTC containing mRNAs accumulate lower level in plants. Since we demonstrated that this is due to a translation-dependent, post-transriptional mRNA degradation mechanisms which could be inhibit with a dominant-negative mutant version of UPF1 protein, we proved that in plants PTC containing mRNAs are degraded by NMD.

4. Our group found that in plants stop codons are identified as PTC if the 3' UTR is unusually long, similar as in yeast. Moreover, we have shown that this effect was size-dependent, mRNAs with longer 3' UTR were more effectively targeted by NMD than transcript with shorter 3' UTR. I have shown, that introns in the 3' UTR could also act as NMD *cis* elements in plants, similar as in mammals. Moreover, the effect of plant introns on mRNA stability is position-dependent. mRNAs carrying an intron 99 nt downstream of the stop codon were targeted by NMD, while transcripts carrying the same intron 28 nt downstream of the stop codon did not trigger NMD. Taken together, both introns and long 3' UTRs operate as *cis*-acting elements to trigger NMD in plants.

5. We have developed an approach (VIGS-NMD system) that allows rapid identification and characterization of NMD *trans* factors by combining our transient NMD assay with *Tobacco rattle virus* (TRV)-mediated gene silencing (VIGS). Following VIGS-mediated knockdown of putative plant NMD factors, NMD activity in silenced plants is assessed by agroinfiltration of GFP-based NMD reporter constructs. Our data suggest that all UPFs (UPF1, UPF2 and UPF3) are required for long 3' UTR-based NMD in plants. UPF1 and UPF2 are also required for intron-based NMD. In contrast, it appears that intron-based NMD is not affected in UPF3-silenced leaves.

6. Intron-based mammalian NMD is mediated by the EJC (Chang, 2007). We hypothesized that intron-based plant NMD is also mediated by the EJC. However, only indirect evidence supports the existence of the EJC in plants. EJC core complex exists as a tetramer of Y14, MAGO, eIF4AIII and Barentz proteins. We have identified putative orthologues of Y14 and MAGO in *N. benthamiana*. Our data suggest that both Y14 and MAGO are required for intron-based NMD but not for long 3' UTR-based NMD, as it was expected. We showed, that Y14 and MAGO form a heterodimer. We failed to identify plant orthologues of the two other components of EJC, so we could not prove the EJC heterotetramer formation in plants. Alternatively, I changed conserved residues involving tetramer formation in Y14 and MAGO. Because these mutations leaded to mutant proteins that are NMD defective but still form heterodimer, we suggested that intron-based NMD is mediated by EJC in plants.

7. Among SMG *trans* factors, we identified putative orthologue of SMG7 in *N. benthamiana*. We found, that both long 3' UTR-based NMD and intron-based NMD require SMG7 factor in plants.

DISCUSSION

1. ANALYSIS OF *POTHOS LATENT VIRUS* ENCODED P14 RNA SILENCING SUPPRESSOR

We demonstrated that *Pothos latent virus* (PoLV, *Tombusviridae, Aureusvirus*) encoded p14 is a dsRNA binding protein that inhibits virus- and transgene-induced silencing. The closest relatives of *Aureusvirus* genera are *Tombusviruses* (*Tombusviridae, Tombusvirus*), which encode the firstly characterized p19 RNA silencing suppressor. Bioinformatic analysis of *Aureusvirus* and *Tombusvirus* suppressors showed, that they evolved from a common ancestor. We found, that p14 is a dsRNA binding protein i*n vitro*. Unexpectedly, p14 binds both longer dsRNA and short sRNA, in contrast with p19 what is a size-specific 21 nt sRNA binding protein (Silhavy, 2002). We suggest that P14 suppresses virus-induced silencing by sequestering double-stranded sRNAs or by delaying the generation of viral sRNAs. Due to the size specific sRNA binding properties of p19, it interferes selectively RNA silencing. In contrast, due to general dsRNA binding properties of p14, it could interact on multiply level of cellular processes. A dsRNA binding protein may interact with tRNAs, rRNAs and other host structural RNAs, inhibiting cell survive before effective virus infection. P14 is translated from 2. subgenomic RNA of the virus (Rubino, 1997). During natural virus infection, amount of 2. subgenomic RNA quickly decreases; 2 day post infection it can be hardly detected. Subsequently, p14 express the beginning of infection. We concluded, that common ancestor of *Tombusvirus* and *Aureusvirus* suppressor could be a general dsRNA binding protein, however, such a protein inhibit not just RNA silencing but also cellular processes and so, it is unfavourable for effective virus infection. It is appealing to speculate that the common ancestor suppressor might have evolved by two ways to reduce the damage to the host. In *Tombusviruses*, it has evolved into a size-specific double-stranded siRNA binding suppressor, while in PoLV (*Aureusvirus*), it could have evolved into a temporally/spatially controlled suppressor.

2. NMD SYSTEM IN PLANTS

Previously, it was proposed that long 3' UTR-based NMD operated in stem eukaryotes (the common ancestors of extant eukaryotes) and has evolved into a more complex intron-based

NMD in vertebrates (Rehwinkel, 2006). Our results support an alternatively model of NMD evolution. As we found that in plants, which are outgroup relatives to fungi and animals, both long 3' UTR- and intron-based NMD operated, we proposed that both NMD systems functioned in stem eukaryotes. This model predicts that the mechanisms of both NMD pathways are conserved between plants and animals. In agreement with this prediction, our results suggest that many NMD *trans* factor is also conserved within eukaryots. UPF1, UPF2 and UPF3 are the core components of the functional NMD complex in yeast as well as in animals (He, 1997; Serin, 2001). We showed, that similar NMD core complex exists in plants. The finding that 3' UTR-located introns elicit NMD in a position-dependent manner and it requires Y14 and MAGO in plants as well as in mammals, suggest that the mechanism of plant and mammalian intron-based NMD is similar which could also operated in stem eukaryotes. Although we have identified plant orthologue of SMG7, its exact function in not known. SMG7 has two (maybe independent) function in mammals; it regulates UPF1 dephosphorylation or/and remobilizes phosphorylated UPF1-containing mRNP complexes into P-bodies, the site of RNA degradation (Fukuhara, 2005; Unterholzner, 2004). It is worth to further analyse which function is conserved or involves in plant NMD.

In summary, our data reveal an unexpected conservation of eukaryotic NMD pathways. Both *cis*-acting elements and *trans*-acting factors similar in most eukaryots; in plants as well as in yeast and mammals. Data about plant NMD system contribute to reconsider previous model of NMD evolution.

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