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Communication

The nuclear cap-binding protein complex is not essential for nonsense-mediated mRNA decay (NMD) in plants

Agnieszka Dzikiewicz-Krawczyk[*], Paulina Piontek, Zofia Szweykowska-Kulińska and Artur Jarmołowski[✉]

Department of Gene Expression, Institute of Molecular Biology and Biotechnology, Faculty of Biology, Adam Mickiewicz University, Poznań, Poland

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In this study we investigated whether in plants, like in mammals, components of the nuclear cap-binding protein complex (CBC) are involved in nonsense-mediated mRNA decay (NMD). We selected several genes producing at least two alternatively spliced mRNA variants: one with a premature termination codon (PTC+) and another without it (PTC–). For each gene the PTC+/PTC– ratio was calculated using RT-PCR and direct sequencing in four *Arabidopsis thaliana* lines: wild type, the NMD mutant *atupf3-1* and two CBC mutants: *cbp20* and *abh1*. Whereas in the NMD mutant the ratios of PTC+/PTC– splice variants were higher than in wild-type plants, the two CBC mutants investigated showed no change in the PTC+/PTC– ratios. Our results suggest that neither CBP20 nor CBP80 is involved in NMD in *A. thaliana*.

Keywords: nonsense-mediated mRNA decay, NMD, nuclear cap-binding protein complex, premature terminatin codon, alternative splicing, mRNA surveillance

INTRODUCTION

Nonsense-mediated mRNA decay (NMD) is an RNA surveillance pathway ensuring that transcripts possessing premature termination codons (PTCs) are recognized and degraded. PTCs can result from mutations, transcription errors or aberrant splicing. Translation of such mRNAs would lead to production of truncated and potentially harmful proteins. It is suggested that apart from guarding mRNA quality, NMD also plays a role in regulating the expression of some genes, especially those whose mRNAs are alternatively spliced (Green, 2003).

NMD is conserved among eukaryotes, but details of its mechanism vary between species. The differences concern the ways of PTC recognition, timing, and cell localization as well as the mechanisms of degradation of PTC-containing transcripts (reviewed in Wagner & Lykke-Andersen, 2002; Maquat, 2004; Conti & Izaurralde, 2005; Lejeune & Maquat, 2005).

NMD has been best studied in mammals and yeast, whereas not much is known about this process in plants. Studies on the identification of main factors involved in NMD and the understanding of details of this mechanism in plants are at an early stage. It has been shown that in plants mRNAs possessing PTCs are subject to degradation *via* the NMD pathway, regardless of whether or not they derive from intron-containing genes (van Hoof & Green, 1996; Petracek *et al.*, 2000; Isshiki *et al.*, 2001). Accumulation of PTC-containing transcripts is reduced if the PTC is located within 70–80% of the mRNA length. Recent experiments of Kertesz *et al.*

[✉]Corresponding author: Artur Jarmołowski, Adam Mickiewicz University, Department of Gene Expression, Umultowska 89, 61-614 Poznań, Poland; tel.: (48) 618 295 959; fax: (48) 618 295 949; e-mail: artjarmo@amu.edu.pl
[*]Present address: Institute of Human Genetics, Polish Academy of Sciences, Strzeszynska 32, 60-479 Poznań, Poland.
Abbreviations: CBC, cap-binding protein complex; NMD, nonsense-mediated mRNA decay; PTC, premature termination codon; UTR, untranslated region.

(2006) indicate that the *cis*-acting signals triggering NMD in plants are: abnormally long 3′ UTR, and introns located in this region. No specific downstream sequence elements (DSE), like the ones described in yeast (Zhang *et al.*, 1995), have been found.

The key factors involved in NMD are three interacting proteins: Upf1, Upf2, and Upf3. Their homologues are present in all eukaryotic organisms studied so far (yeast, nematodes, flies, and mammals). In plants the role of Upf1 (Arciga-Reyes *et al.*, 2006) and Upf3 (Hori & Watanabe, 2005) has been confirmed, but other proteins potentially involved in NMD have not been studied yet. In order to identify the factors essential for this process in plants, the most convenient way to start with is to examine proteins whose role in NMD has been described in other organisms.

In this study we tested whether two proteins of the nuclear cap-binding protein complex (CBC) (Kmieciak *et al.*, 2002), CBP20 and CBP80/ABH1, are involved in NMD in *Arabidopsis thaliana.* The accepted model for NMD in mammals assumes that this process takes places during the pioneer round of translation, when the mRNA is bound by CBC at its 5′ end (Ishigaki *et al.*, 2001, Chiu *et al.*, 2004). It has also been reported that CBP80 interacts directly with Upf1 and promotes interactions between Upf1 and Upf2. Furthermore, silencing the expression of CBP80 in human cells leads to increased accumulation of PTC-containing transcripts (Hosoda *et al.*, 2005). Based on these clues we decided to examine whether in plants, similarly to mammals, CBP20 and CBP80 are involved in NMD.

MATERIALS AND METHODS

Plant materials. The following plant lines were used: *Arabidopsis thaliana* ecotype Columbia wild type (wt), the *atupf3-1* mutant (SALK_025175), seeds of homozygous plants received from Y. Watanabe (Hori & Watanabe, 2005), the T-DNA insertion mutant *cbp20* (Papp *et al.*, 2004) provided by C. Koncz, and the T-DNA insertion mutant *abh1* (Hugouvieux *et al.*, 2001) provided by J. Schroeder. Before sowing, seeds were subjected to surface sterilization with the solution consisting of 100 ml 5.25% household cleaning agent ACE (Procter & Gamble) and 3 ml of hydrochloric acid.

Seeds were planted on Jiffy-7 (Jiffy, Batavia, IL, USA) and plants were grown under long day conditions (16 h light, 8 h dark) in an MLR-350H Sanyo phytotron.

RNA purification and RT-PCR. Total RNA was purified from 20-day-old plants using Trizol reagent (Invitrogen) according to the manufacturer's protocol. RNA samples (6 μg) were freed from DNA contamination by DNase treatment (Promega) (1 U/1 μg RNA), purified by phenol/chloroform extraction and used to synthesize cDNA using MMLV reverse transcriptase (Promega) and oligo-dT as a primer (Fermentas) according to the enzyme producer. Twenty nanograms of cDNA was used for amplification. PCR was performed in a 20 μl mixture with *Taq* polymerase (1 U/reaction) (Fermentas) and appropriate primer sets (Table 1). All primers were synthesized by the Laboratory of DNA Sequencing and Oligonucleotide Synthesis at the Institute of Biochemistry and Biophysics PAS (Warszawa, Poland).

Evaluation of PTC+/PTC-mRNA ratios by RT-PCR direct sequencing. The PTC+/PTC– mRNA ratios were evaluated by direct sequencing of the RT-PCR products using the same primer pairs as in the PCR reactions. Sequencing was performed on the Applied Biosystems 3130xl Genetic Analyzer using Big Dye Terminator v. 3.1. Chromatograms obtained from sequencing of RT-PCR products were analyzed with Chromas Lite v. 2.01 (Technelysium Pty Ltd). This method was previously shown to be semi-quantitative (Hori & Watanabe, 2005). For each gene used in this study the peak heights of one hundred bases were measured for each splicing variant. The PTC+/PTC– ratios were calculated for corresponding bases and mean values were determined. For all plant types the peak heights were measured for the same nucleotides in a given gene.

Table 1. Primers used in RT-PCR reactions

Name	Orientation	Gene	Sequence
NMD-1F	Forward	At5g07910	5′AGTTTCAGCTGATGGAAGGA3′
NMD-1R	Reverse	At5g07910	5′AAGTAGAATCGTCATCTCAATATAAG3′
NMD-2F	Forward	At3g63340	5′CCCTGTAGCAACTGTACGCTTC3′
NMD-2R	Reverse	At3g63340	5′TTCTTCAGCACATAGCTTGAG3′
NMD-3F	Forward	At1g51340	5′TGTGTAACTCTCTCCGCGTC3′
NMD-3R	Reverse	At1g51340	5′TGTCCCAATCCGCCAGAATCC3′
NMD-5F	Forward	At5g62760	5′CCTCATCGATCTACTCGTCC3′
NMD-5R	Reverse	At5g62760	5′TCCATGTCCATATCCACCTCC3′
NMD-6F	Forward	At2g45670	5′AAGAATGCTGTGCATGAAATAAAG3′
NMD-6R	Reverse	At2g45670	5′CTGCTTACATGGAATAGCGAC3′

RESULTS AND DISCUSSION

In order to examine whether the plant CBC complex is involved in NMD, ratios of the PTC+ to PTC– mRNA forms were calculated for five genes in the *abh1* and *cbp20* mutants and compared with those in the wild type plant. The *atupf3-1* mutant was used as a positive control since it was shown previously that the disruption of the *A. thaliana UPF3* gene led to significant accumulation of PTC+ mRNA variants (Hori & Watanabe, 2005).

In this study we used the genes analyzed earlier by Hori and Watanabe (2005). Five genes were chosen whose pre-mRNAs are alternatively spliced giving two mRNA variants: one with a PTC (PTC+), and another without it (PTC–). In addition, in the At3g63340 gene, there are two alternative splice sites (giving, together with the constitutively spliced variant, three mature transcripts): one (A) does not create a frameshift or a PTC, whereas the second one (B) causes a shift of 4 bp leading to a PTC+ variant (see Table 2).

In order to determine the PTC+/PTC– ratios, total RNA was purified from wild type plants and the *abh1, cbp20* and *atupf3-1* T-DNA insertion mutants. The cDNAs obtained after reverse transcription were used as templates for PCR reaction with specific primer pairs. The PCR products were sequenced and the PTC+/PTC– ratios calculated by measuring peak heights of corresponding bases on the chromatograms. It had been shown earlier that this method is semi-quantitative (Hori & Watanabe, 2005). The experiment was done for two independent biological samples.

Two genes were excluded from the final analysis. In our test the At5g07910 gene did not produce a PTC+ form in any of the plant types tested, not even in the *atupf3-1* mutant. On the other hand, in the case of At5g62760 the PTC+ form was dominant in all the plant types tested (PTC+/PTC– ratio: 1.7–2.0; with no significant differences between wild type and mutants), even in the wild type (Fig. 1). Analysis of the remaining three genes showed that, as expected, the PTC+/PTC– ratios were significantly increased in the *atupf3-1* mutant when compared to those in the wild type plant. However, there were no differences in the relative levels of the PTC-containing transcripts between the *abh1* and *cbp20* mutants and wild type plants (Fig. 2). Taken together, these results show that the nuclear cap-binding protein complex (CBC) is not involved in NMD in plants. The increased relative levels of PTC+ mRNAs in the *atupf3-1* mutant (our positive control) make our results reliable. So yet another difference in the NMD mechanism between animals and plants has emerged from this research. It has been shown in mammals (Hosoda *et al.*, 2005) that CBP80 may play an active role in NMD, as silencing its expression increases the level of transcripts with PTCs. However, the results presented here indicate clearly that neither CBP80 nor CBP20 is involved in NMD in *A. thaliana*.

One gene we investigated – At5g62760 – may raise some interest. In our hands, in all the plant types examined the PTC+ form derived from this gene dominated over the PTC– variant, in contrast to what was described by Hori and Watanabe (2005). In their work the PTC+/PTC– ratio for At5g62760 was >1, as expected, only in the *atupf3-1* mutant, not in the wild type plant. However, At5g62760 varies from other genes used in the study in that the alternative splicing event in pre-mRNA processing is exon skipping which is relatively rare in *A. thaliana* (less than 10% of all alternative splicing events described in this species) (Wang & Brendel, 2006). Nevertheless, the PTC+ form derived from this gene can be easily found in databases, so it does not seem uncommon. It seems plausible that under certain conditions the splice site skipping of the 6th exon is preferred and the protein translated from such mRNA is functional. Still, the question remains why the PTC+ form could be dominant — is it because it is produced in such a high quantity that in spite of being degraded in the NMD process (which does not lead to complete decay of all PTC-containing mRNAs) its level remains higher than that of the PTC– form? Or perhaps this transcript contains some inherent features making it immune to NMD. If the second explanation is true, further studies on transcripts of At5g62760 could shed some light on the mechanism of NMD in plants by characterizing features protecting mRNAs from it.

In the light of our results it will be interesting to examine whether in plants, like in mammals, NMD takes place during the pioneer round of translation when mRNAs are bound by CBC, or if PTC-containing transcripts are also recognized and degraded when bound by eIF4E, i.e. during the steady-state translation. Another possibility is that in plants NMD can take place only on mRNAs bound by eIF4E.

Table 2. Genes used in the study

Gene	Protein function	PTC+ form frameshift
At5g07910	Leucin-rich repeat protein family	+8 bp
At3g63340	Protein phosphatase 2C-related/ PP2C-related	p. A: +3 bp (no PTC) p. B: –4 bp
At1g51340	MATE efflux family protein	–19 bp
At5g62760	Nuclear protein ZAP-related	–56 bp
At2g45670	Calcineurin B subunit-related	+14 bp

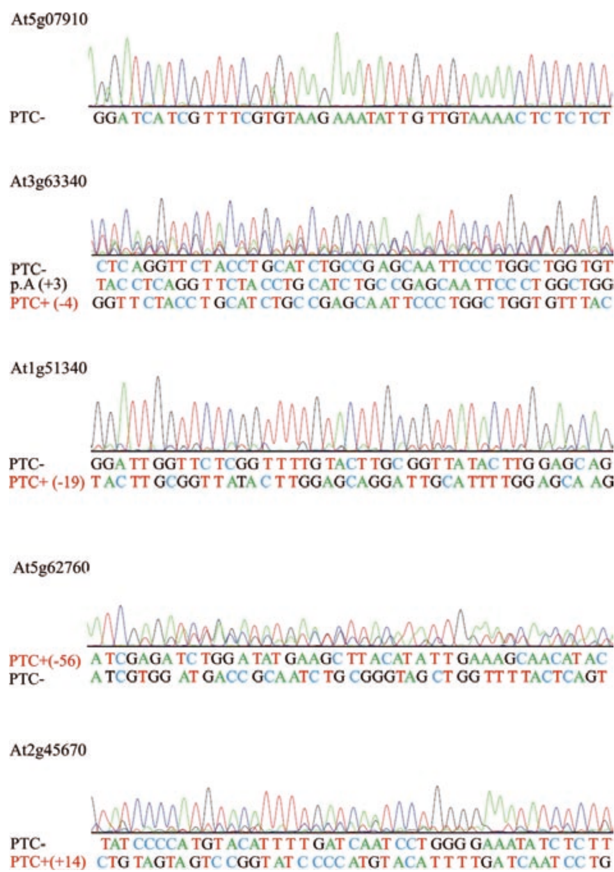

Figure 1. Sample chromatograms from sequencing of the PCR products using appropriate primer pairs.
Apart from the At5g07910 gene, in every case products of alternative splicing can be seen as minor peaks shifted by a given number of nucleotides. In the case of the At5g62760 gene the PTC+ form dominates over the PTC– one in all plant types tested.

This study, by showing that plant CBC proteins are not crucial for NMD, provides novel data about nonsense-mediated decay in plants and shows another difference in the mechanism of nonsense mediated decay among eukaryotes. At the same time the results indicate directions for further studies that may broaden our knowledge of the mechanism of NMD in plants.

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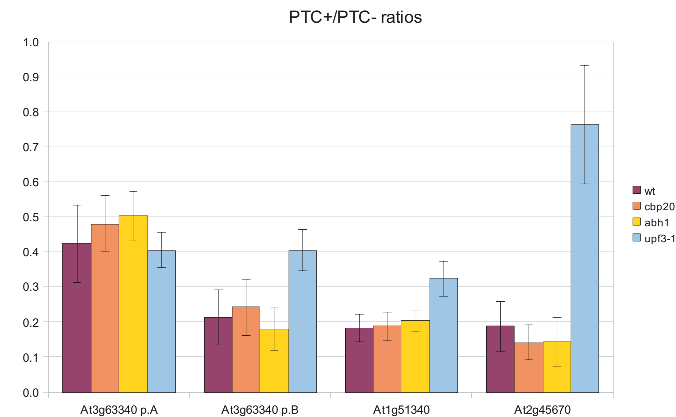

Figure 2. PTC+/PTC– ratios for wild type plant and *cbp20*, *abh1* and *atupf3-1* mutants.
Mean values and standard deviations are shown, calculated by measuring peak heights of 100 bases for each gene. Two independent experiments were carried out.