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A conserved DYW domain of the pentatricopeptide repeat protein possesses a novel endoribonuclease activity

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Abstract **Many plant pentatricopeptide repeat (PPR) proteins are known to contain a highly conserved C-terminal DYW domain whose function is unknown. Recently, the DYW domain has been proposed to play a role in RNA editing in plant organelles. To address this possibility, we prepared recombinant DYW proteins and tested their cytidine deaminase activity. However, we could not detect any activity in the assays we used. Instead, we found that the recombinant DYW domains possessed endoribonuclease activity and cleaved before adenosine residues in the RNA molecule. Some DYW-containing PPR proteins may catalyze site-specific cleavage of target RNA species.**

Keywords: PPR protein; DYW domain; RNA editing; Endoribonuclease; RNase

1. Introduction

An unusually large gene family encoding the pentatricopeptide repeat (PPR) proteins exists in land plants, compared with a small gene family in animals and yeast [1,2]. For instance, *Arabidopsis thaliana*, *Oryza sativa*, and the moss *Physcomitrella patens* contain 450, 477, and 103 genes, respectively encoding PPR proteins [3]. Most PPR proteins are predicted to localize in mitochondria or plastids, and have been implicated in the control of organelle gene expression [2] since several PPR proteins were demonstrated to be involved in site-specific cleavage, splicing, or RNA editing for targeted organellar transcripts [4–6]. PPR motifs probably act as sequence-specific RNA binding proteins, and may recruit some catalytic factors for RNA cleavage, splicing or RNA editing, etc.

The PPR proteins are structurally divided into P and PLS subfamilies. The P subfamily is composed of only canonical PPR (P) motifs and the PLS subfamily consists of repeated units of the classic PPR (P) motif, and longer (L) and shorter (S) non-canonical PPR motifs [2,3]. The P and PLS-type PPR proteins occupy approximately half of all PPR proteins in *A. thaliana* and *O. sativa*. The remaining ones are PLS proteins with a highly conserved C-terminal domain, 87 of which in *A. thaliana* contain the DYW domain. This domain was named because of its characteristic terminal tripeptide (Asp-Tyr-Trp) and has not been found in any other proteins or in any organisms apart from land plants. The DYW domain contains a conserved region composed of an αβα secondary structure, which includes invariant residues that match the active site of cytidine deaminases from bacteria, plant, animals, and yeast [7]. Cytidine deaminases are zinc-dependent enzymes containing a motif corresponding to the active site, C/HxExx...xPCxxC [8]. The DYW domain contains the CxxCH motif of the cytochrome *c* family heme-binding signature [9], which suggests that it may have a certain catalytic activity. However, the actual function of these motifs is completely unknown.

Neither RNA editing nor DYW domains could be identified in algae or the marchantiid liverworts. There is an intriguing correlation between the presence of nuclear DYW genes and organelle RNA editing among embryophytes. These observations provide a hypothesis that the DYW domains are responsible for RNA editing in plant organelles and catalyze RNA editing [7,10]. Therefore, we investigated the function of the DYW domain. Here, we show that this domain can act as an endoribonuclease.

2. Materials and methods

2.1. Transient expression of At2g02980-green fluorescent protein (GFP) fusion protein in tobacco protoplasts

A DNA fragment (1809 base pair (bp)) encoding the full-length At2g02980 protein was amplified from *A. thaliana* ecotype Columbia-0 genomic DNA by polymerase chain reaction (PCR) using primers 2g02980(sal)-F and 2g02980F(nco)-R (see Supplemental Table 1). The amplified DNA fragment was digested by *Sal*I-*Nco*I and ligated into *Sal*I-*Nco*I digested CaMV35S-sGFP (S65T)-nos3′ [11]. The resultant plasmid p02980-GFP was introduced into *Nicotiana tabacum* Bright Yellow 4 leaf cell protoplasts as described previously [12].

2.2. Production of recombinant proteins

The DNA sequence encoding a 109 amino acid (aa) C-terminal DYW domain of At2g02980 was PCR-amplified from *A. thaliana* genomic DNA using primers 02980DYW-F(eco) and 02980-R(sal). The sequence encoding tobacco cp28 protein was PCR-amplified from plasmid pNS28 [13] using primers 28N-F(eco) and 28N-R(sal). The DNA sequence encoding a 108 aa C-terminal region containing the DYW domain of Os05g30710 was amplified from *O. sativa* genomic DNA using primers os05g30710-F and os05g30710-R. The DNA fragment for T-28DYW was prepared by combining by PCR (with primers 28N-F(eco) and 02980-R(sal)), the two partial fragments obtained by amplification using either primers (28N-F(eco) and 28DYW-R) and pNS28 [13], or primers (28DYW-F and 02980-R(sal)) and *A. thaliana*

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Abbreviations: aa, amino acid(s); bp, base pair(s); nt, nucleotide(s); GFP, green fluorescent protein; PCR, polymerase chain reaction; PPR, pentatricopeptide repeat; Trx, thioredoxin; RNase, ribonuclease

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genomic DNA, as described previously [14]. The DNA fragment for T-DYW_M was generated likewise by two successive PCR reactions, using primers 02980DYW-F(eco), 02980M2-R, 02980M2-F and 02980-R(sal) and *A. thaliana* genomic DNA.

The PCR products were inserted in-frame into pBAD/Thio-TOPO (Invitrogen) and the plasmids pT-DYW, pT-osDYW, pT-28, and pT-28DYW were obtained. The recombinant proteins were expressed in *Escherichia coli* LMG194 as a fusion with thioredoxin at its N-terminus and six histidine residues at the C-terminus, and purified by binding to Probond N resin (Invitrogen). They were dialyzed against a solution (20 mM HEPES-NaOH, pH 7.9, 60 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 3 mM dithiothreitol (DTT), and 17% glycerol).

2.3. Preparation of RNA and DNA probes

A 538 bp 5′ translated region of the plastid *ndhB* gene was amplified from *A. thaliana* genomic DNA (10 ng) using primers ndhB-F and ndhB-R. ndhB-F contains a promoter sequence for T7 RNA polymerase and ndhB-R has a sequence forming a stem-loop sequence to prevent attack by $3' \rightarrow 5'$ exonucleases at the 3′ terminus of RNA. The PCR product (named NB500 DNA) was *in vitro* transcribed by T7 RNA polymerase to produce a 514 nucleotide (nt) of [α-^{32}P]-CTP or [α-^{32}P]-UTP labeled RNA (named NB500 RNA). The ^{32}P-labeled NB500 DNA was obtained by PCR amplification using [α-^{32}P]-dCTP and the same primer set. Single-stranded (ss) NB500 DNAs were obtained by heat denaturing ^{32}P-labeled double-stranded (ds) NB500 DNA at 90 °C for 3 min and subsequent cooling at 4 °C. ^{32}P-labeled RNA or DNA was gel-purified as described [15].

2.4. Assay of cytidine deaminase activity

[^{32}P-CTP]-labeled NB500 RNA (1 fmol, 0.05 nM, 10 000 cpm) was incubated for 30 min at 25 °C with the recombinant protein in 10 mM Tris–HCl, pH 7.9, 30 mM KCl, 6 mM $MgCl_2$, 2 mM ATP, 1 mM $ZnCl_2$ 2 mM DTT, and 8% glycerol [16]. Then, the RNA was extracted and digested at 37 °C for 3 h into 5′ mononucleotides by 1 μg of nuclease P1 and 120 units of S1 nuclease (Takara) in the presence of 50 mM ammonium acetate (pH 4.8). The resultant mononucleotides were separated on a cellulose thin-layer chromatography plate using isopropanol/hydrochloride/water (70:15:15). The separated ^{32}P-mononucleotides were visualized by autoradiography.

2.5. RNA cleavage assay

Internal [^{32}P-UTP]-labeled NB500 RNA (1 fmol, 0.05 nM, 10 000 cpm) was denatured by heating to 70 °C for 2 min, and transferred to 25 °C. Then, the ^{32}P-labeled RNA and the recombinant protein were incubated for 30 min at 25 °C in 20 μl of reaction mixture (10 mM Tris–HCl, pH 7.9, 30 mM KCl, 6 mM $MgCl_2$, 25 mM EDTA, 2 mM DTT, 8% glycerol). After incubation, the ^{32}P-RNA was extracted by phenol/chloroform followed by ethanol precipitation, and then analyzed on a 6% polyacrylamide gel containing 6 M urea.

2.6. Primer extension analysis for identification of 5′ ends of RNA fragments

To determine the cleavage site by T-DYW protein, the cleavage reactions were carried out using 1 μg T-DYW protein and non-radiolabeled NB500 RNA (10 fmol) for 15 or 30 min at 25 °C. The resultant RNA was subjected to primer extension analysis [17], using the ^{32}P-labeled primer barrier-R (5′-CCCATAGGGATTTAGGTGACACTC-3′). The sequence ladder was obtained using NB500 DNA, the same primer, and the thermo-sequencing kit (GE healthcare).

3. Results

3.1. Characterization of DYW domain-containing PPR protein (At2g02980)

In this study we selected several DYW domain-containing PPR proteins for analysis. Among them, we chose the *Arabidopsis* PPR protein At2g02980, which consists of 603 aa residues with 10 PPR motifs and C-terminal E, E+ and DYW domains, because a transferred DNA (T-DNA) insertion mutant line Salk_008983 [18] displayed a severe dwarf phenotype (Supplemental Fig. 1). We next analyzed whether At2g02980 protein is localized in either plastids or mitochondria, or both. Transient expression assays in tobacco leaf cell protoplasts showed that At2g02980 is a mitochondrial protein (Supplemental Fig. 2). This suggested that disruption of the *At2g02980* gene impaired mitochondrial function (probably respiration), and resulted in retarded development of plant seedlings.

To identify the target transcripts for the At2g02980 PPR protein, we performed northern blot analysis of total cellular RNA from the wild-type and T-DNA tag line plants using 23 mitochondrial protein gene-specific probes. However, we could not detect any aberrant transcripts in the T-DNA tag line (data not shown).

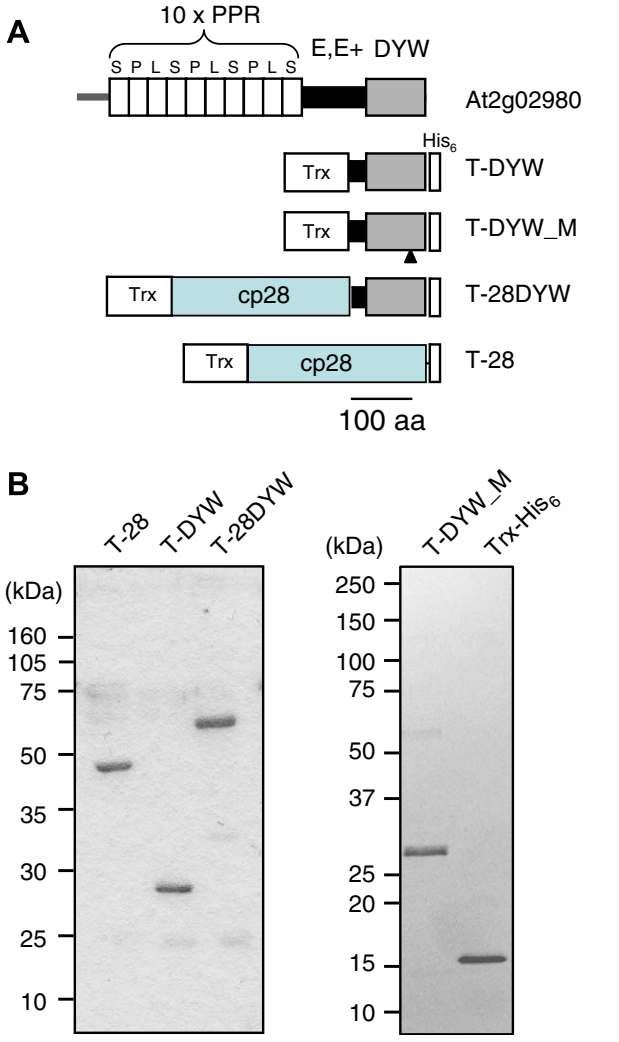

Fig. 1. Structure and expression of the recombinant proteins. (A) Predicted domain structure of At2g02980 protein and various recombinant proteins. PPR motifs are indicated as canonical PPR (P), PPR-like S, and L according to [2]. The C-terminal E, E+ motifs and DYW domain are indicated by a filled box and a gray box, respectively. The position of the mutated cysteine residues in putative heme-binding signature of DYW domain is shown by a filled triangle. The recombinant proteins were expressed as fused protein with thioredoxin (Trx) at the N-terminus and His_6 tag at the C-terminus. (B) The recombinant proteins were separated by SDS–PAGE and stained with Coomassie Brilliant Blue.

3.2. The DYW domain has a novel endoribonuclease activity

To characterize the DYW domain of At2g02980, we made four recombinant proteins, T-DYW, T-DYW_M, T-28 and T-28DYW (Fig. 1). T-28 was composed of tobacco chloroplast RNA binding protein cp28 [13] and in T-28DYW cp28 was fused to the DYW domain (Fig. 1A). All the recombinant proteins were expressed as soluble proteins, and therefore purified under native conditions (Fig. 1B).

We first tested cytidine deaminase activity of T-DYW and T-28DYW by using [^{32}P CTP]-labeled NB500 RNA. If some cytidines converted to uridines in the RNA, a spot of [^{32}P]-labeled uridines could be detected by thin-layer chromatography. However, the predicted spot of uridines was not detected under our experimental conditions (Fig. 2A). Instead, we observed that the RNA probe was rapidly degraded to produce discrete fragments (Fig. 2B). This activity depended on the concentration of T-DYW. In contrast, T-28 protein did not digest the RNA although T-28DYW weakly digested it (Fig. 2B). Cleavage of the RNA by T-DYW was strongly suppressed by addition of increasing amounts of T-28 protein (Fig. 2C). T-DYW protein digested the RNA but not ssDNA and dsDNA (Fig. 3), which were digested by ribonuclease (RNase)-free DNase I under the same condition. These results indicate that T-DYW is an endoribonuclease.

In general, RNase activity depends on Mg ions [19]. However, RNase activity of T-DYW was not enhanced even by the addition of 1 to 10 mM $MgCl_2$ (Fig. 4A). In contrast, EDTA enhanced the RNase activity of T-DYW at an optimal concentration of 25 mM (Fig. 4B). However, higher concentrations of EDTA (100–200 mM) inhibited its activity. ATP and Zn

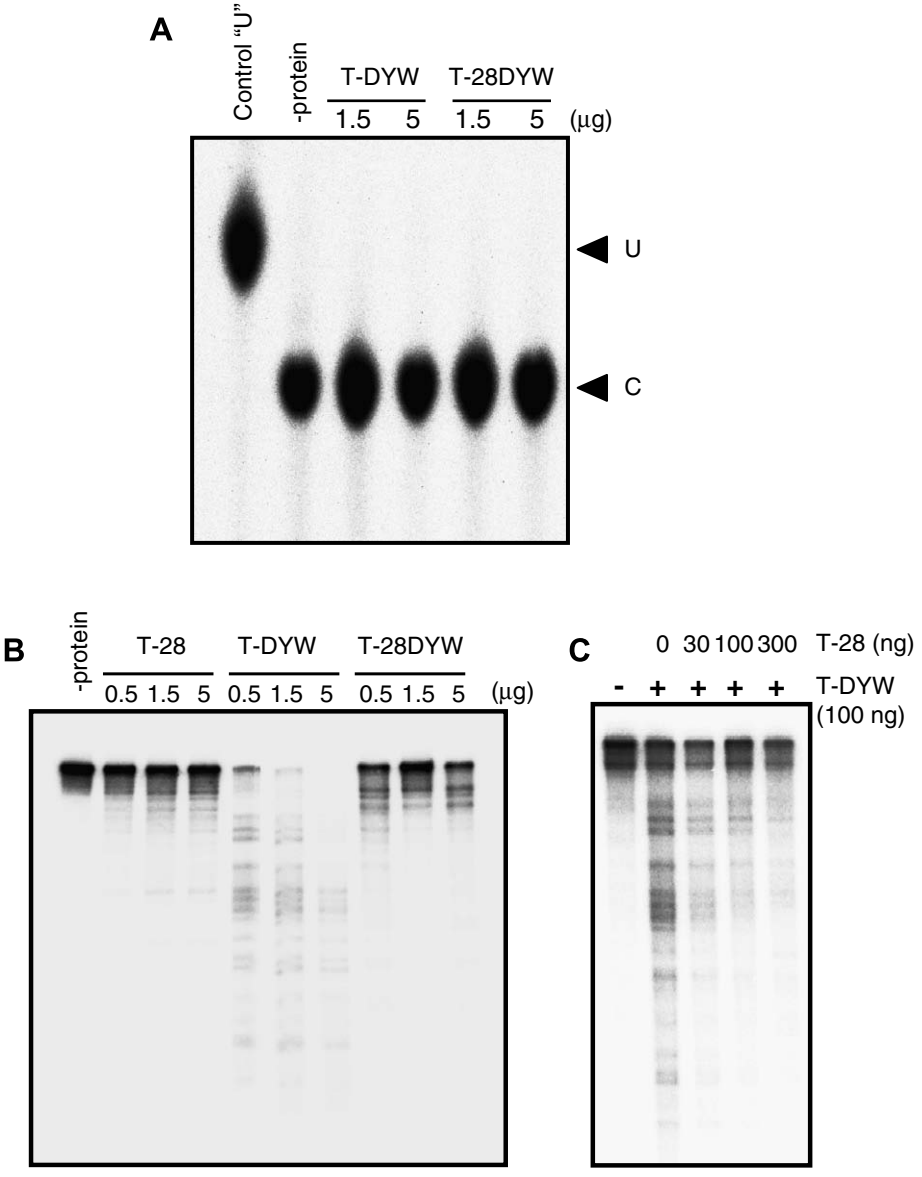

Fig. 2. Detection of biochemical activities of the recombinant proteins. (A) Assay for detection of cytidine deaminase activity of the recombinant T-DYW and T-28DYW proteins. [α-^{32}P CTP]-labeled NB500 RNA (10 000 cpm) was incubated for 30 min with the indicated proteins (1.5 or 5 μg) or without protein (minus protein), then digested into mononucleotides, and separated by thin-layer chromatography. The control "U" spot was prepared from [α-^{32}P UTP]-labeled NB500 RNA. (B) RNA cleavage assay. Indicated amount of the recombinant protein was incubated with [^{32}P]-labeled NB500 RNA in the presence of 6 mM $MgCl_2$ and 25 mM EDTA, and the RNA was extracted and analyzed on a 6% polyacrylamide gel containing 6 M urea. (C) The T-DYW protein (100 ng) was incubated with NB500 RNA in the presence of the indicated amounts of T-28.

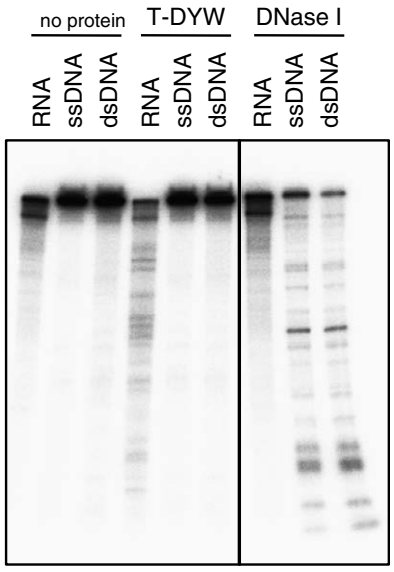

Fig. 3. The T-DYW protein digests RNA but not DNAs. The T-DYW protein (100 ng) or DNase I (0.5 unit) was incubated with ^{32}P-labeled RNA, ssDNA or dsDNA prepared from NB500 DNA.

ion did not affect the RNA cleavage activity of the T-DYW protein, whether EDTA was added, or not (Supplemental Fig. 3).

To investigate whether other DYW domains possess RNase activity or not, we analyzed the DYW of rice PPR protein Os05g30710 (664 aa), whose DYW domain has significant similarity to that of At2g02980. The rice protein contains 13 PPR motifs and was predicted to be localized in plastids. We produced recombinant T-osDYW and tested the activity. The T-osDYW protein also displayed RNase activity (Supplemental Fig. 4).

3.3. A CxxCH motif in the DYW domain is required for endoribonuclease activity

The DYW domain contains the cytochrome *c* family heme-binding site signature (CxxCH). To investigate the role of this signature we made the recombinant protein T-DYW_M whose CxxCH motif was mutated to GxxGH and tested its RNase activity. Mutation of this motif resulted in a significant reduction (loss) of cleavage activity (Fig. 5). This indicated that the CxxCH motif is required for endoribonuclease activity of the DYW domain.

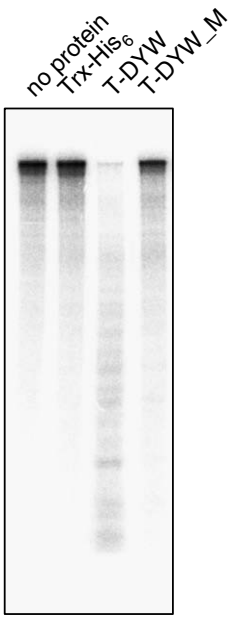

Fig. 5. Mutation of the conserved cysteine residues in T-DYW resulted in a loss of RNA cleavage activity. Respective protein (Trx-His_6, T-DYW or T-DYW_M; 100 ng) was incubated with ^{32}P-labeled NB500 RNA in the presence of 6 mM $MgCl_2$ and 25 mM EDTA.

3.4. Identification of endonucleolytic cleavage sites

The discrete RNA fragments produced by T-DYW suggest that it recognizes definite primary sequence(s) and/or secondary structure(s) of RNA. Therefore, we attempted to identify the cleavage sites by primer extension analysis. The termini of the primer extended products were mapped at 288A,

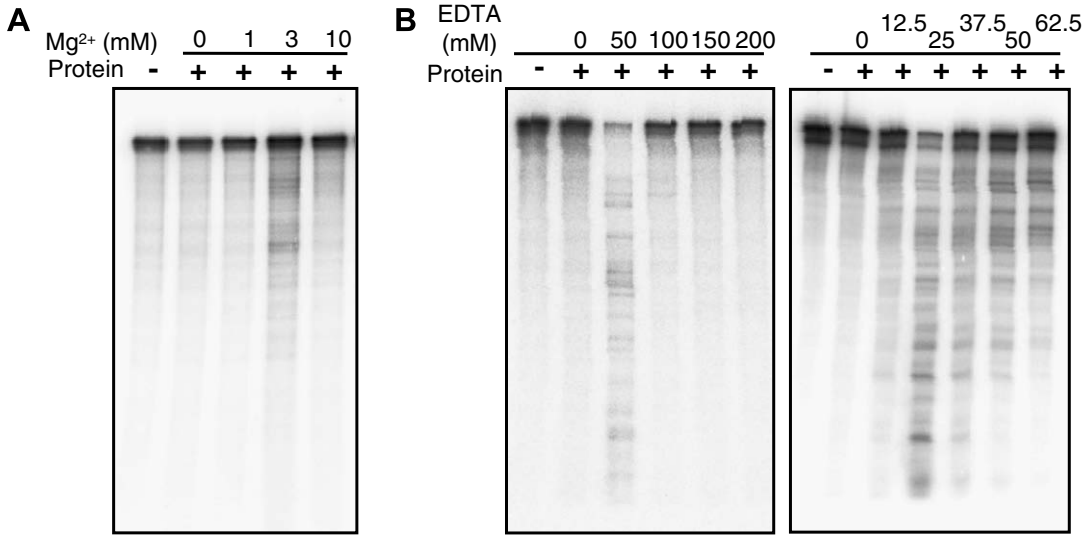

Fig. 4. Effect of EDTA and Mg^{2+} on RNA cleavage activity. (A) T-DYW protein dialyzed against a buffer without Mg^{2+} was incubated with the NB500 RNA in the presence of the indicated concentrations of Mg^{2+} but without EDTA. (B) T-DYW protein (100 ng) was incubated with [^{32}P]-labeled NB500 RNA in the presence of 6 mM $MgCl_2$ and various concentrations of EDTA (left panel, 0–200 mM and right panel, 0–62.5 mM).

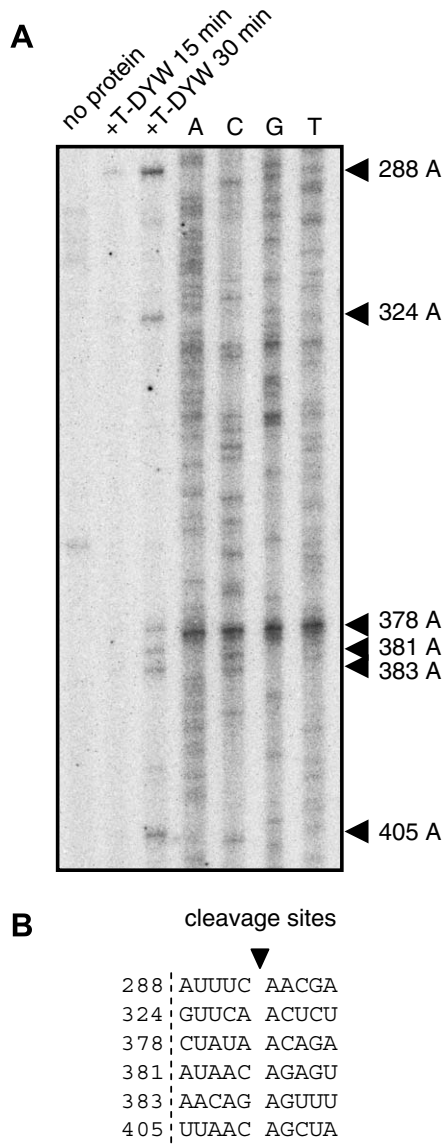

Fig. 6. Mapping of the cleavage sites produced by T-DYW protein. (A) T-DYW protein (1 μg) and non-radiolabeled NB500 RNA (10 fmol) were incubated for 15 or 30 min at 25 °C. The resulting RNA fragments were subjected to primer extension analysis. The sequence ladder was obtained using NB500 DNA and the same primer. Primer-extended products are indicated by arrows and the numbers refer to the positions from the 5′ end of NB500 RNA. (B) RNA sequences flanking the cleavage sites are shown.

324A, 378A, 381A, 383A and 405A relative to the 5' end of NB500 RNA (Fig. 6A). This indicated that the T-DYW cleaved before adenosine residues of the RNA probe. There are no consensus sequences in the flanking region of cleavage sites (Fig. 6B).

4. Discussion

In this study, cytidine deaminase activity of the DYW domain that we initially expected was not detected under our assay conditions. However, this does not mean that the DYW domain would not have such activity under other conditions, with other substrates, or in the presence of other polypeptides. Instead, we found that the DYW domain possessed RNase activity that cleaved before adenosines in the RNA molecule. The recombinant DYW proteins contained additional C-terminal 30 aa residues consisting of V5 epitope and six histidines after the natural C-terminal tripeptide DYW. Therefore, we cannot exclude the possibility that tag sequences might abolish correct folding or function of the DYW domain.

RNase T1 cleaves regularly and exclusively after G while RNase U2 cleaves after adenosine residues. RNase A cleaves exclusively after pyrimidines [19]. Therefore, the DYW domain is a novel type of endoribonuclease. In addition, its activity is enhanced in the presence of EDTA. This is quite unique to the DYW RNase.

Although the T-DYW domain cleaved at multiple sites, it is possible that At2g02980 plays a role in site-specific RNA cleavage in mitochondria. The PPR motifs of At2g02980 protein may recognize sequences of target RNA molecules and the DYW domain might cleave at a specific site. In this study, the target RNA molecule of At2g02980 PPR protein was not identified. Perhaps if the actual target RNA and the recombinant full-sized At2g02980 PPR protein were incubated together, site-specific cleavage(s) might be observed.

In plant mitochondria, site-specific endonucleolytic cleavage by RNase P and RNase Z is required for 5′ and 3′ end formation of mRNAs [20]. The rice fertility restoration factor, Rf-1a, is a member of the PPR protein family and promotes cleavage of the *atp6-orf78* transcript [21,22]. The *Arabidopsis* protein OTP43 is required for *trans*-splicing of the mitochondrial *nad1* intron 1 and belongs to the P subfamily of PPR proteins [23]. These PPR proteins do not contain the DYW domain. To date, there are no reports on the mitochondrial DYW-containing PPR proteins that are involved in RNA cleavage of pre-mRNAs. In plastids, the *Arabidopsis* CRR2, a PPR protein with DYW domain, was well characterized to be responsible for the site-specific intergenic cleavage between the *rps7* and *ndhB* pre-mRNA [24]. Therefore, it is possible that the CRR2 acts as a site-specific endoribonuclease.

In the present study, both an *Arabidopsis* and a rice DYW domain exhibited endoribonuclease activity. This suggests that additional PPR-DYW proteins may act as RNA cleavage factors in addition to *Arabidopsis* CRR2. Analysis of additional members of the 87-member *Arabidopsis* PPR-DYW family and the 90-member rice PPR-DYW family [3] will be necessary to conclude whether or not endoribonuclease activity is a common feature of DYW-domain-containing proteins.

The fact that inhibition of the RNase activity of DYW domain by chloroplast RNA-binding protein cp28 was observed (Fig. 2 B and C) is significant because gene-specific RNA cleavage occurs in the presence of various RNA-binding proteins [25]. In plant mitochondria, RNA-binding proteins similar to cp28 are present [26]. Such general organelle RNA-binding proteins have strong affinity to RNAs and therefore could inhibit the binding of PPR proteins to the target RNAs. Site-specific cleavage of the target RNA may occur by cooperation of PPR-DYW proteins and organelle RNA-binding proteins.

The reason why EDTA affected the RNase activity of the DYW domain is unknown, but the relatively small range of effective EDTA concentrations (20–60 mM) may provide a useful tool when exploiting DYW-domain-containing proteins for applied purposes. In this study, RNase activity was detected when excess amounts of the T-DYW protein were incu-

bated with an RNA substrate. The weak activity may be due to the absence of PPR repeats in the recombinant DYW protein we produced. It may be possible to combine PPR motifs with various DYW domains to create artificial sequence-specific endoribonucleases. Such possible applications will first require further detailed biochemical analysis of DYW domains and their enzymatic activities.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.11.017.

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