Differential substrate recognition by isozymes of plant protein-only Ribonuclease P

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ABSTRACT

Ribonuclease P (RNase P) catalyzes the cleavage of leader sequences from precursor tRNA (pre-tRNA). Typically, these enzymes are ribonucleic protein complexes that are found in all domains of life. However, a new class of RNase P has been discovered that is composed entirely of protein, termed protein-only RNase P (PRORP). To investigate the molecular determinants of PRORP substrate recognition, we measured the binding affinities and cleavage kinetics of *Arabidopsis* PRORP1 for varied pre-tRNA substrates. This analysis revealed that PRORP1 does not make significant contacts within the trailer or beyond N₋₁ of the leader, indicating that this enzyme recognizes primarily the tRNA body. To determine the extent to which sequence variation within the tRNA body modulates substrate selectivity and to provide insight into the evolution and function of PRORP enzymes, we measured the reactivity of the three *Arabidopsis* PRORP isozymes (PRORP1–3) with four pre-tRNA substrates. A 13-fold range in catalytic efficiencies (10^4 – 10^5 M⁻¹ s⁻¹) was observed, demonstrating moderate selectivity for pre-tRNA substrates. Although PRORPs bind the different pre-tRNA species with affinities varying by as much as 100-fold, the three isozymes have similar affinities for a given pre-tRNA, suggesting similar binding modes. However, PRORP isozymes have varying degrees of cleavage fidelity, which is dependent on the pre-tRNA species and the presence of a 3'-discriminator base. This work defines molecular determinants of PRORP substrate recognition that provides insight into this new class of RNA processing enzymes.

Keywords: PRORP; RNase P; fidelity; isozymes; tRNA

INTRODUCTION

Transfer RNAs (tRNAs) are transcribed as precursors (pre-tRNA) that contain extra nucleotides flanking the 5' and 3' ends. Removal of these extraneous sequences is critical for tRNA function, thus the enzymes responsible for tRNA maturation are essential. RNase P is the endonuclease responsible for catalyzing the 5' end maturation of pre-tRNA and is found in all domains of life (Walker and Engelke 2006). RNase P enzymes are extremely diverse with regard to their macromolecular composition (Howard et al. 2013). In Bacteria, Archaea, and some eukaryotic lineages, pre-tRNA cleavage is catalyzed by a conserved RNA-based RNase P (ribozyme) that associates with a number of different protein cofactors (1–10, depending on the domain) (Lechner et al. 2015). However, the majority of eukaryotic lineages are predicted to use a protein-only form of RNase P (PRORP) to catalyze pre-tRNA maturation within organelles (mitochondria

and chloroplast) and, depending on the lineage, within the nucleus (Gobert et al. 2010; Lechner et al. 2015). PRORPs were first discovered in human mitochondria, where they process mitochondrial encoded pre-tRNAs (Holzmann et al. 2008). Human mitochondrial RNase P requires three protein subunits for efficient catalysis: a nuclease (MRPP3/human PRORP), a tRNA methyltransferase (TRMT10C/MRPP1), and a dehydrogenase (SDR5C1/MRPP2). SDR5C1 and TRMT10C form a complex and are proposed to play a scaffolding role in pre-tRNA maturation catalyzed by human PRORP (Vilardo et al. 2012; Vilardo and Rossmann 2015). In contrast to the mammalian enzyme, the recombinant PRORPs from plants, some protists, and algae do not require additional proteins for efficient catalysis, offering a simpler model system to understand the function of this new class

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of nuclease (Gobert et al. 2010; Lai et al. 2011; Taschner et al. 2012).

While mammals retain an RNA-based RNase P in their nucleus, bioinformatic studies suggested that land plants lack a catalytic RNA component for RNase P activity (Hartmann and Hartmann 2003). Consistent with this, the prototypical land plant *Arabidopsis thaliana* possesses three nuclear-encoded PRORP enzymes (PRORP1, 2, and 3) (Fig. 1A; Goldfarb et al. 2012; Gutmann et al. 2012). PRORP1 is essential and is localized to the mitochondria and chloroplasts (Gobert et al. 2010; Gutmann et al. 2012). PRORP2 and 3 are localized to the nucleus where they play essential but redundant roles; knockout of both PRORP2 and PRORP3 is lethal; however, knockout of either one is not (Gobert et al. 2010; Gutmann et al. 2012; Zhou et al. 2015). Similar to *Arabidopsis*, the moss *Physcomitrella patens* contains three PRORP isoforms and no apparent catalytic RNA component for the RNA-dependent RNase P (Sugita et al. 2014). In contrast to *Arabidopsis*, *P. patens* localizes two PRORP enzymes to the mitochondria and chloroplast and one to the nucleus (Sugita et al. 2014). Thus, PRORP enzymes may have replaced the ancient RNA-based RNase P in *A. thaliana* and *P. patens*, processing tRNA transcripts in the chloroplast, mitochondria, and nucleus. However, the extent to which these isoforms vary in substrate specificity and the molecular interactions that confer substrate specificity of these important enzymes remains largely unknown.

PRORP enzymes contain three domains: an N-terminal pentatricopeptide repeat (PPR) domain, a central Zn-binding domain, and a Nedd4-BP1, YacP nuclease (NYN) domain (Fig. 1; Howard et al. 2012). The PPR domain contains five PPR or PPR-like motifs, which are helix–turn–helix motifs found in tandem. This domain has been proposed to interact with pre-tRNA and enhance binding affinity (Howard et al. 2012; Gobert et al. 2013; Imai et al. 2014). The largest sequence variation among the PRORPs is found

in the PPR domains and thus differences in substrate recognition may lie within this region. The central domain binds a Zn^{2+} ion and structurally links the PPR and metallonuclease domains. The NYN-metallonuclease domain catalyzes phosphodiester bond hydrolysis and contains four conserved aspartates important for binding catalytic metal ions and catalysis (Howard et al. 2012, 2015). Both the protein and RNA-based RNase P enzymes are proposed to use a two-metal ion mechanism for catalysis (Steitz and Steitz 1993; Howard et al. 2015). Despite this similarity, RNA-based RNase P enzymes have higher catalytic efficiency under in vitro conditions than PRORP enzymes (Howard et al. 2013). The sequence identity among the *A. thaliana* PRORPs is highest in the metallonuclease domain. PRORP2 and 3 are most similar (80% identity and 88% similarity); the percent identity and similarity between PRORP1 and 2 are 48% and 65% and between PRORP1 and 3 are 49% and 65%, respectively.

To provide insight into the evolution and function of PRORP enzymes, we measured substrate specificities, equilibrium binding affinities, and cleavage fidelities for varied pre-tRNA substrates. Previous studies have identified the importance of the elbow region of tRNA for PRORP1 substrate recognition (Gobert et al. 2013; Imai et al. 2014). However, recognition of the tRNA leader and trailer by PRORP is largely unknown. Our data demonstrate no dependence on the trailer or leader length beyond the first nucleotide (N_{-1}). This is in contrast to bacterial RNA-based RNase P that makes significant interactions with the leader and trailer sequences of pre-tRNA (Crary et al. 1998; Niranjankumari et al. 1998; Reiter et al. 2010; Sun et al. 2010). However, the pre-tRNA sequence alters the binding affinity (≤ 40 -fold) and, hence, the catalytic efficiency of PRORP cleavage. The three PRORP isoforms have comparable catalytic efficiencies for a given pre-tRNA suggesting similar, but not identical, substrate selectivity. However, PRORP isoforms

FIGURE 1. (A) *A. thaliana* encodes three PRORP enzymes, PRORP1, 2, and 3. N-terminal truncations of PRORP1 ($\Delta 76$) and PRORP3 ($\Delta 9$) were used in this study. PRORP enzymes contain three domains: PPR (red), central-Zn binding (yellow), and NYN (blue) domains. The N-terminal green region represents the proposed localization sequences: PRORP1 contains a mitochondrial targeting peptide (1–48, predicted by the TargetP 1.1 algorithm [Emanuelsson et al. 2007]), while PRORP2 and 3 contain canonical bipartite nuclear localization signals (H/R-RSR-R/H-X₉-K-K-K-K) (Lange et al. 2007). The pink region represents a plant-specific insert in the gene sequence. (B) The crystal structure of PRORP1 (PDB 4G24) with the domains colored as in A.

have varying degrees of cleavage fidelity, which is dependent on the pre-tRNA species and the presence of a 3'-discriminator base. This work defines molecular determinants of PRORP substrate recognition that provides insight into this new class of RNA processing enzymes.

RESULTS

PRORP1 substrate recognition has little dependence on leader and trailer length

The binding affinity of *Bacillus subtilis* RNase P for pre-tRNA has a significant dependence on pre-tRNA leader length, with the pre-tRNA affinity increasing ~50-fold when the leader length is increased from one to five nucleotides (Hsieh and Fierke 2009). To determine whether PRORP1 shares a similar dependence on leader length, we measured the binding affinity and single-turnover (STO) cleavage rate constant for *B. subtilis* pre-tRNA^{ASP} lacking the 3'-CCA sequence. This substrate was previously used to interrogate both the mechanism of PRORP (Howard et al. 2015) and leader interactions with *B. subtilis* RNase P (Crary et al. 1998; Rueda et al. 2005; Hsieh and Fierke 2009). This allows a direct comparison between the recognition modes of PRORP1 and bacterial RNase P.

A fluorescence anisotropy binding assay was used to measure the equilibrium dissociation constant (K_D) of PRORP1 binding to 5' fluorescein-labeled *B. subtilis* pre-tRNAs with varying leader lengths while maintaining a discriminator base on the 3' end (Fig. 2A). Increasing the pre-tRNA leader length from 1 to 14 nt has little effect on PRORP1 binding affinity (Table 1; Fig. 2B), with K_D values ranging between 100 and 310 nM. The observed binding trend is independent of the presence of the 5'-fluorescein label on the 5' end of pre-tRNA (Supplemental Fig. S1). In contrast, the binding affinity of the mature tRNA product (0 nt leader) is ~30-fold weaker than for pre-tRNA. These data suggest that PRORP1 does not significantly recognize the leader past the first nucleotide (N_{-1}).

To examine the effect of leader length on PRORP1 cleavage activity, the STO rate constant (k_{obs}) was measured using a gel-based assay. The observed rate constant under these conditions is proposed to mainly reflect the chemical cleavage step (Howard et al. 2015). In all cases, the leader length was consistent with cleavage at the correct site. The STO rate constant for *B. subtilis* pre-tRNA with varying leader lengths was generally uniform (Table 1; Fig. 2C), with the ex-

ception of the 1- and 2-nt leader substrates, which have approximately four- and eightfold increased STO rate constant compared to 14:1 (leader nucleotide length: trailer nucleotide length) pre-tRNA, respectively. This increased reactivity may be due to catalytic enhancement imparted by the positioning of the 5' guanosine and fluorescein. The cleavage rate constant of the 1:1 pre-tRNA may depend on the sequence at both the -1 nt and the discriminatory base, as suggested by the slower STO cleavage rate of an unmodified U-tRNA (Supplemental Fig. S3). Nonetheless, these data indicate that PRORP1 can efficiently bind and catalyze removal of 1-nt leader *B. subtilis* pre-tRNA, further demonstrating that PRORP1 does not significantly contact the leader sequence beyond N_{-1} .

To determine whether the trailer is an important determinant for PRORP1 recognition, we assayed the STO activity and binding affinity of PRORP1 with *A. thaliana* mitochondrial encoded Cys-Mito pre-tRNA (Fig. 3) containing a 24-nt long trailer and a 5-nt long leader (5:24). A Cys-Mito pre-tRNA substrate was used for these studies because extension of the *B. subtilis* pre-tRNA trailer to 20 nt resulted in significant STO biphasic kinetics (data not shown), potentially as a result of the pre-tRNA adopting an alternative structure (Bhaskaran et al. 2014). Variation of the Cys-Mito trailer length does not alter the binding affinity ($K_D = 65 \pm 5$ and 70 ± 10 nM for 5:1 and 5:24, respectively, under standard binding conditions with the exception of 100 mM NaCl) or

FIGURE 2. Binding affinity and STO activity of PRORP1 for varying leader lengths of *B. subtilis* pre-tRNA. (A) Predicted secondary structures of *B. subtilis* pre-tRNAs with varying leader lengths used in this study. All substrates were prepared using in vitro transcription. The 5' end is labeled with fluorescein (Fl). The arrow indicates the RNase P cleavage site. (B) Representative binding isotherms for 0:1 (mature tRNA with a discriminator base at the 3' end) (closed square), 1:1 (closed circle), and 14:1 (open circle). Values for STO rate constants and K_D 's are reported in Table 1. (C) Representative STO timecourses for PRORP1 catalyzed *B. subtilis* pre-tRNA cleavage under standard reaction conditions; 1:1 (closed circle), 5:1 (closed square), and 14:1 (open circle).

TABLE 1. Dissociation constant (K_D) and STO observed rate constant (k_{obs}) for PRORP1 with *B. subtilis* pre-tRNA containing varying leader lengths

pre-tRNA	K_D (nM) ^a	Fold ^b	k_{obs} (s ⁻¹) ^c	Fold ^b
0	3400 ± 400	34	—	—
1	150 ± 60	1.5	0.078 ± 0.003	3.9
2	310 ± 20	3.1	0.15 ± 0.02	7.8
3	140 ± 40	1.4	0.032 ± 0.001	1.6
4	150 ± 40	1.5	0.025 ± 0.001	1.3
5	190 ± 60	1.9	0.025 ± 0.001	1.3
10	100 ± 50	1	0.025 ± 0.001	1.3
14	100 ± 30	1	0.02 ± 0.001	1

^aMeasured as described in the legend of Figure 2 and Materials and Methods. The mean and standard deviation is reported from two independent determinations.

^bAs compared to pre-tRNA with a 14-nt leader.

^cMeasured as described in the legend of Figure 2 and Materials and Methods. The standard error from fitting is reported.

STO cleavage rate constant ($k_{\text{obs}} = 0.037 \pm 0.002$ and 0.033 ± 0.001 for 5:1 and 5:24, respectively), suggesting that PRORP1 does not make significant contacts with the 3' trailer. Taken together, these data suggest that the leader (past N₋₁) and trailer sequences are not important determinants of molecu-

lar recognition by PRORP1, therefore PRORP1 mainly recognizes the tRNA body.

PRORP reactivity with model substrates reveals the importance of the D-arm in recognition

To determine the regions within the tRNA body that are important for PRORP recognition, we created two truncated versions of pre-tRNA Cys-Mito. These model substrates include an RNA lacking an anti-codon arm (Δ AC), and an RNA that connects the acceptor arm and T ψ C-arm to form a stem-loop (SL), thereby removing the D- and anticodon arms (Fig. 3). Both were labeled at the 5' end with fluorescein and cleavage catalyzed by PRORP1 was assessed under STO conditions. PRORP1 catalyzed removal of the 5' leader from the Δ AC substrate with an observed rate constant similar to pre-tRNA, $k_{\text{obs}} = 0.037 \pm 0.002$ and 0.023 ± 0.005 s⁻¹ for Cys-Mito pre-tRNA and Δ AC, respectively. PRORP1 binding affinity for the Δ AC and pre-tRNA substrates are similar: $K_D = 70 \pm 10$ and 65 ± 5 nM, respectively (under standard binding conditions with the exception of 100 mM NaCl). This result is consistent with a previous observation that PRORPs can cleave t-element RNA, tRNA-like structures that lack an anticodon stem-loop (Gobert et al. 2010;

FIGURE 3. (A) Predicted secondary structures of *A. thaliana* pre-tRNA substrates used in this study. All substrates were prepared using in vitro transcription. Cys-Mito and Phe-Chlor substrates are derived from the organellar genomes (Cys-mitochondrial and Phe-chloroplast), and Cys-nuclear and Phe-nuclear are from the nuclear genome. The pre-tRNAs contain 5-nt leaders labeled with fluorescein at the 5' end and a discriminator base at the 3' end. (B) Model substrates. Predicted secondary structures of two model substrates, Δ AC and SL, derived from the mitochondrial pre-tRNA^{Cys} sequence. The SL substrate connects the acceptor stem helix and T ψ C-arm.

Gutmann et al. 2012). The SL RNA was cleaved >1000-fold slower than Cys-Mito pre-tRNA ($k_{\text{obs}} < 3.3 \times 10^{-5} \text{ s}^{-1}$). This result indicates that the D-arm of pre-tRNA is critical for substrate binding and/or cleavage by PRORP1.

***Arabidopsis* PRORP isozymes display differential catalytic efficiencies with four pre-tRNAs**

Given that the PRORP1 substrate recognition relies on interactions with the tRNA body, differences in the nucleotide sequence of tRNA species could potentially alter recognition and catalytic efficiencies. To explore the range of reactivity, we measured the steady-state (multiple-turnover, MTO) kinetic parameters of the three *A. thaliana* PRORPs with four pre-tRNAs. The pre-tRNAs assayed are: two nuclear-encoded pre-tRNAs (Cys-Nuc and Phe-Nuc) and two organellar-encoded pre-tRNAs (Cys-Mito and Phe-Chlor) (Fig. 3). We used a real-time fluorescence anisotropy assay to measure the steady-state kinetic parameters for PRORP-catalyzed pre-tRNA hydrolysis (Table 2; Figure 4A; Liu et al. 2014). A comparison of the catalytic efficiencies (k_{cat}/K_M values) reveals that PRORP1 has the highest activity, with values two- to 13-fold higher for a given substrate than PRORP2 and 3 (Fig. 4B). The kinetic parameter k_{cat}/K_M is a measure of the productive associations between enzyme and substrate and sets a lower limit for the second-order rate constant for substrate binding (k_{on}). PRORP1 is most selective for the Phe-Chlor substrate ($k_{\text{cat}}/K_M = 4.1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$) relative to PRORP2 and 3, representing a nine- and 13-fold enhancement, respectively. Each isozyme reacts fastest with a different pre-tRNA: PRORP1 with Phe-Chlor, PRORP2 with Phe-Nuc, and PRORP3 with Cys-Mito. The values of the turnover number (k_{cat}) for cleavage catalyzed by PRORP1

and 2 vary little (approximately twofold) between substrates. However, PRORP3 catalyzes cleavage of the Cys-Nuc substrate nearly eightfold slower than Phe-Nuc pre-tRNA. These data indicate that sequence variation within the tRNA body can modulate substrate specificity, albeit within a range of 10^4 – $10^5 \text{ M}^{-1} \text{ s}^{-1}$.

PRORPs bind individual pre-tRNAs similarly

To further explore determinants of substrate recognition, we measured the dissociation constants (K_D) of PRORP1, 2, and 3 for the four *A. thaliana* pre-tRNA substrates using the fluorescence anisotropy binding assay (Fig. 5A; Liu et al. 2014). The affinity of the PRORPs for these substrates varies by as much as 100-fold (60–6000 nM) (Table 3; Fig. 5B), demonstrating significant discrimination between the substrates. However, comparison of the dissociation constants reveals that all three PRORPs have comparable binding affinities for a given substrate (less than fourfold difference). All three PRORPs demonstrate the weakest affinity for the Cys-Nuc pre-tRNA and the highest affinity for the Phe-Chlor pre-tRNA. Thus, the PRORP enzymes use similar binding modes for pre-tRNA substrate recognition.

Product release is not rate-limiting for MTO catalysis by PRORP

The kinetic parameters measured under MTO conditions include all steps in the PRORP kinetic mechanism. To begin dissecting the contribution of discrete steps in the mechanism (e.g., substrate cleavage, product release, etc.) to the progression of the reaction, we measured the STO kinetics of PRORP1, 2, and 3 for catalysis of pre-tRNA cleavage.

TABLE 2. Kinetic parameters for pre-tRNA cleavage catalyzed by *A. thaliana* PRORP1, 2, and 3

Enzyme	Pre-tRNA	Single-turnover ^a	Multiple-turnover ^b		
		$k_{\text{obs}} (\text{s}^{-1})^c$	$k_{\text{cat}} (\text{s}^{-1})$	$K_M (\text{nM})$	$k_{\text{cat}}/K_M (\text{mM}^{-1}\text{s}^{-1})$
PRORP1	Cys-Mito	0.037 ± 0.002	0.062 ± 0.005	670 ± 230	96 ± 10
	Phe-Chlor	0.035 ± 0.002	0.042 ± 0.005	140 ± 50	410 ± 60
	Cys-Nuc	0.037 ± 0.003	0.040 ± 0.002	550 ± 50	73 ± 5
	Phe-Nuc	0.078 ± 0.003	0.035 ± 0.003	160 ± 50	220 ± 60
PRORP2	Cys-Mito	0.013 ± 0.003	0.013 ± 0.002	340 ± 60	37 ± 6
	Phe-Chlor	0.018 ± 0.003	0.015 ± 0.002	340 ± 100	45 ± 10
	Cys-Nuc	0.027 ± 0.002	0.030 ± 0.002	940 ± 130	32 ± 3
	Phe-Nuc	0.035 ± 0.002	0.023 ± 0.002	250 ± 50	95 ± 16
PRORP3	Cys-Mito	0.023 ± 0.002	0.022 ± 0.002	430 ± 30	53 ± 3
	Phe-Chlor	0.023 ± 0.002	0.013 ± 0.002	440 ± 50	32 ± 3
	Cys-Nuc	0.030 ± 0.002	0.008 ± 0.002	420 ± 100	17 ± 4
	Phe-Nuc	0.072 ± 0.003	0.062 ± 0.023	2000 ± 850	37 ± 8

^aReactions contained 5 μM PRORP, 30 nM pre-tRNA, 30 mM MOPS pH 7.8, 150 mM NaCl, 1 mM TCEP, and 1 mM MgCl_2 at 25°C. The standard error from fitting is reported.

^bAs described in the legend of Figure 4 and Materials and Methods. The standard error from fitting is reported.

^cBecause both correct and incorrect cleavage products are catalyzed with some substrates (See Fig. 6; Supplemental Fig. S2), k_{max} represents the single-exponential fit to the time course of total product ($C_0 + M_{-1}$) formation. The multiple-turnover kinetics similarly reflect total product formation.

FIGURE 4. Multiple-turnover cleavage of pre-tRNA catalyzed by PRORPs. (A) The substrate dependence of the initial velocity ($v_0/[E]$) for cleavage of Cys-Mito pre-tRNA catalyzed by PRORP1 (closed circle), PRORP2 (closed square), and PRORP3 (open circle). Reactions were performed under standard reaction conditions. (B) Bar graph comparing the catalytic efficiencies (k_{cat}/K_M) of the three PRORP enzymes with the four pre-tRNA substrates. The error bars represent the standard error from fitting.

In contrast to the MTO k_{cat} , the STO rate constant k_{obs} includes only the steps in the kinetic mechanism prior to and including substrate cleavage. For all three PRORPs, the observed STO rate constants (k_{obs}) range between 0.02–0.07 s^{-1} (Table 2), regardless of the substrate assayed. The observed rate constant for total product formation ($C_0 + M_{-1}$) is reported for pre-tRNA substrates that are significantly miscleaved (see following section).

For *B. subtilis* RNase P, a significant difference (~300-fold) between the STO and MTO rate constants previously revealed that product release is the rate-limiting step in the reaction (Beebe and Fierke 1994). However, the PRORP MTO k_{cat} values are within twofold of the STO k_{obs} values, suggesting that the rate-limiting step at saturating PRORP is a step prior to product dissociation. The one exception is the difference between the k_{obs} and k_{cat} values for PRORP3 catalyzing cleavage of Cys-Nuc pre-tRNA (k_{obs} is 3.6-fold greater than k_{cat}), suggesting that product release is partially rate limiting for this enzyme/substrate pair.

PRORPs have varying cleavage fidelities

Gel analysis of the STO cleavage assays indicate two distinct product bands in all PRORP-catalyzed reactions with the Phe-Nuc substrate, and for PRORP2 and 3 reactions with the Cys-Mito and Cys-Nuc substrates (Fig. 6; Supplemental Fig. S2). To further investigate the miscleavage, assay products were separated by high resolution urea-PAGE and compared to *B. subtilis* RNase P-catalyzed cleavage of pre-tRNAs (Fig. 6). This analysis revealed that the products represent the correct (5 nt, C_0) and incorrect (4 nt, M_{-1}) cleavage products (Fig. 6). Additionally, all enzymes cleave *B. subtilis* pre-tRNA^{Asp} correctly, indicating that the miscleavage observed with other substrates is not a result of nuclease

contamination. *B. subtilis* RNase P cleaves all four *A. thaliana* pre-tRNAs correctly; only a distinct 5-nt product band (<5% miscleaved product) is observed. In contrast, the PRORP enzymes have variable cleavage fidelities, with PRORP3 displaying the lowest cleavage fidelity among the PRORPs (Fig. 6; Supplemental Table S1). Furthermore, the nuclear-localized PRORP2 and PRORP3 significantly miscleaved the Cys-Mito and Cys-Nuc substrates whereas the organellar PRORP1 did not (<5%), demonstrating differences in cleavage site selection between the nuclear and organellar PRORPs (Fig. 6; Supplemental Table S1).

PRORP-catalyzed miscleavage of pre-tRNA between the –2 and –1 nt (M_{-1}) generates a product with a 1-nt leader, a potential substrate for PRORP. Since we are assaying cleavage using a 5' labeled pre-tRNA, we cannot determine whether the miscleaved product is further processed by PRORP. Thus, we performed primer extension assays on tRNA at various time-points taken during STO cleavage reactions catalyzed by PRORP1 and 3 of Phe-Nuc pre-tRNA (the substrate miscleaved by all isozymes) (Supplemental Fig. S3). These data demonstrate that the miscleaved product is further processed by PRORP to generate mature tRNA (Supplemental Fig. S3). However, the observed rate constant for PRORP catalyzed removal of the 1-nt leader is decreased (~ 0.0006 – $0.001 s^{-1}$) (Supplemental Fig. S3).

3' discriminator base can contribute to PRORP cleavage fidelity

One feature of the Phe-Nuc substrate that could lead to the observed miscleavage by all three PRORP enzymes is the formation of a base pair between the adenine discriminator base and the uridine base in the N_{-1} position of the leader (Fig. 3), which would extend the acceptor stem helix creating a 4-nt leader. Consistent with this hypothesis, the three pre-tRNA

FIGURE 5. (A) Representative fluorescence anisotropy binding isotherms for varying concentrations of PRORP1 and 20 nM fluorescein-labeled pre-tRNA (Cys-Mito [closed circle], Phe-Chlor [closed square], Cys-Nuc [open circle] and Phe-Nuc [open square]) in 20 mM MOPS pH 7.8, 300 mM NaCl, 1 mM TCEP, and 1 mM CaCl₂. (B) Bar graph comparing the binding affinity (K_D) of the PRORP isozymes for four pre-tRNAs. The error bars represent the standard error from fitting.

TABLE 3. Dissociation constants (K_D in nM) for PRORP1, 2, and 3 binding to pre-tRNAs^a

Enzyme	Pre-tRNA			
	Cys-Mito	Phe-Chlor	Cys-Nuc	Phe-Nuc
PRORP1	510 ± 120	60 ± 10	2300 ± 300	330 ± 60
PRORP2	350 ± 70	140 ± 10	6100 ± 2100	350 ± 40
PRORP3	300 ± 70	220 ± 30	1500 ± 200	380 ± 50

^aMeasured as described in the legend of Figure 5 and Materials and Methods. The standard error from fitting is reported.

substrates that can form an extra base pair (U/A) between the discriminator base and the N_{-1} leader position (Phe-Nuc, Cys-Mito, and Cys-Nuc) exhibit miscleavage, albeit to varying extents depending on the PRORP (Fig. 6). To test this hypothesis, we assessed the ability of PRORPs to correctly cleave three different Phe-Nuc pre-tRNAs variants possessing 5-nt leaders and variable 3' ends: with (5:1) or without (5:0) a 3' discriminator base and with an extended 20 nt trailer sequence (5:20) (Fig. 7). Removal of the discriminator base (5:0) from Phe-Nuc pre-tRNA eliminated the miscleavage catalyzed by all of the PRORP enzymes, whereas addition of a longer trailer sequence had no significant effect on miscleavage (Fig. 7). Similarly, removal of the discriminator base from the Cys-Mito pre-tRNA, to delete a potential A–U base pair with N_{-1} , significantly reduced miscleavage catalyzed by PRORP3 (Supplemental Fig. S4).

Taken together, these data provide evidence that the presence of a base pair between N_{-1} and the discriminator base can engender miscleavage by PRORPs, possibly due to extension of the acceptor stem. However, the potential to form this base pair does not guarantee miscleavage; the Cys-Mito and Phe-Chlor substrates, which contain a potential base pair between the N_{-1} and the discriminator base, are not significantly miscleaved by PRORP1 or 2. Thus, other determinants including sequence context, stability of the pre-tRNA structure, and differences in PRORP substrate recognition must also influence cleavage fidelity.

DISCUSSION

To define the molecular determinants of PRORP recognition, we examined the reactivity of PRORP1 with model substrates and pre-tRNAs with varying leader and trailer lengths. This analysis revealed that PRORP1 makes little or no catalytically important interactions with the trailer or with the leader past

the first nucleotide (Table 1; Fig. 2). This is in contrast to bacterial RNase P where pre-tRNA binding affinity increases ~50-fold upon increasing the leader from 1–5 nt (Hsieh and Fierke 2009). Thus, PRORP enzymes do not share a major determinant of molecular recognition utilized by bacterial RNase P. Furthermore, PRORP1 and *B. subtilis* RNase P bind mature tRNA ~30- and 400-fold weaker than pre-tRNA (leader length ≥ 4 nts), respectively (Crary et al. 1998). This is consistent with PRORP1 making fewer interactions with the leader than *B. subtilis* RNase P. Similar to PRORP1, recent binding studies with *A. thaliana* nuclear pre-tRNA^{Gly} suggest PRORP2 does not require long leader (≤8 nt) and trailer (1 nt) lengths for tight binding (Karasik et al. 2016). PRORPs efficiently catalyze removal of leader sequences from Cys-Mito tRNA lacking an anti-codon stem-loop (ΔAC) but not from a model substrate lacking the anticodon stem-loop and D-arm (SL substrate). However, bacterial RNase P can catalyze processing of minimal stem-loop model substrates (Brännvall et al. 2007), demonstrating a more important role for the interaction between the D-arm and PRORP compared to bacterial RNase P. Thus far, the available data reveal that the molecular determinants of PRORP1 substrate recognition lie within the elbow of the tRNA body and the N_{-1} nucleotide of the leader. Given the similarities in binding affinities and kinetic parameters of the PRORP isozymes for pre-tRNAs, it is likely that PRORP2 and 3 also share these determinants.

FIGURE 6. PAGE analysis of reaction products from RNase P-catalyzed STC cleavage of pre-tRNA. Of note, 30 nM pre-tRNA substrate was incubated with either 5 μM PRORP for 30 min under standard assay conditions or 1.8 μM *B. subtilis* RNase P for 60 min under conditions described in Materials and Methods. The pre-tRNA substrate in each reaction is indicated above the wells. The enzyme in each lane is indicated as follows: (C) no enzyme control, (B.s.) *B. subtilis* RNase P, (1) PRORP1, (2) PRORP2, (3) PRORP3, and (L) alkaline hydrolysis ladder. The normalized percent miscleaved (% M_{-1}) is indicated below the lane number at the bottom of the gel. Closed arrows indicate the correct cleavage product (5 nt, C_0) and open arrows indicate miscleaved product (4 nt, M_{-1}).

FIGURE 7. Miscleavage of pre-tRNA catalyzed by PRORP is alleviated by removal of the discriminator base. Of note, 30 nM Phe-Nuc pre-tRNA substrates (5:20, 5:1, and 5:0) were incubated with 5 μ M PRORP1, 2, or 3 for 30 min under standard reaction conditions. Products were resolved on a 20% gel. Closed arrows indicate the correct cleavage product (5 nt, C_0) and open arrows indicate miscleaved product (4 nt, M_{-1}). The normalized percent miscleaved (% M_{-1}) is indicated below the lane number at the bottom of the gel. In each case, miscleavage is decreased for the substrate lacking the discriminator base (5:0).

The detailed kinetic comparison of *A. thaliana* PRORP enzymes reveals both similarities and differences. In general, the three *A. thaliana* PRORPs catalyze 5' end cleavage with comparable catalytic efficiencies (10^4 – 10^5 $M^{-1} s^{-1}$) and pre-tRNA binding affinity. This suggests that the differentially localized PRORP isozymes are not selective for pre-tRNAs of different organellar origin. The STO cleavage rate constants among the PRORP isozymes are consistent with a previous study using a bacterial pre-tRNA^{Gly} substrate, where the observed rate constants vary between 0.04 and 0.13 s^{-1} (Pavlova et al. 2012). Despite these similarities, our data indicate that there is a subset of pre-tRNAs that are more efficiently processed by specific PRORP isozymes. For example, PRORP1 has a ~ 10 -fold higher k_{cat}/K_M value for cleaving Phe-Chlor pre-tRNA compared to PRORP2 and 3. Nucleotide variations in the D- and T ψ C-loops may contribute to preferential cleavage and recognition by PRORP1. However, more detailed studies are needed to determine the molecular interactions that confer this enhanced reactivity.

While PRORPs bind and cleave substrates with similar K_D values and cleavage rate constants, they exhibit unexpected

differences in cleavage fidelity. For instance, PRORP3 catalyzes the miscleavage of Cys-Mito, Cys-Nuc, and Phe-Nuc pre-tRNA to a greater extent than PRORP1 and PRORP2 (Fig. 6). Removal of the discriminator base from Cys-Mito or Phe-Nuc pre-tRNAs results in increased fidelity for all three enzymes, suggesting that the discriminator base engenders miscleavage (Fig. 7). Base-pairing between the discriminator nucleotide and the N_{-1} nucleotide of the leader, extending the acceptor stem by one base pair, could account for the observed miscleavage. The greater fidelity observed with PRORP1 could originate from specific interactions with the N_{-1} of the leader and/or interactions with the discriminator base. These data suggest a difference in substrate recognition among the PRORPs with regards to cleavage site selection. While the frequency of PRORP-catalyzed pre-tRNA miscleavage in vivo is unknown, PRORP catalyzes phosphodiester bond hydrolysis between -1 nt and $+1$ nt (correct) and between -2 nt and -1 nt (miscleavage) both in vivo and in vitro with the atypically processed plant mitochondrial pre-tRNA^{His} (Placido et al. 2010). Furthermore, PRORPs can correct miscleavage by catalyzing the removal of the miscleaved 1-nt leader, albeit slower than the rate constant for the initial cleavage step (Supplemental Fig. S3). This step is likely slow due to base-pairing between the -1 nt and the discriminator base. Cleavage fidelity in vivo could be enhanced by RNA binding proteins that decrease incorrect base-pairing or that stabilize RNA structure.

While all three PRORP enzymes catalyze miscleavage of the Phe-Nuc pre-tRNA (Fig. 6), *B. subtilis* RNase P catalyzes only the correct product from the same substrate. This observation suggests that PRORP enzymes use different criteria to recognize the substrate cleavage site than bacterial RNase P. Bacterial RNase P interacts with the elbow of tRNA (nucleotides within the T ψ C and D-loops), the acceptor stem, nucleotides within the leader sequence, and the CCA of the 3' end of pre-tRNA, though all are not required for accurate cleavage (Zahler et al. 2005; Reiter et al. 2010). Interactions that could increase fidelity of bacterial RNase P compared to PRORP in cleavage of the Phe-Nuc substrate are the base-pairing between the RNA component and both the N_{-1} uridine and the 3' end of pre-tRNA, leading to splaying of the 3' and 5' ends in the bound complex (Zahler et al. 2003; Reiter et al. 2010). Future experiments examining the nucleotide identity near the cleavage site, including the discriminator base, 1 nt/72 nt base pair, and 3'-CCA, will further define PRORP cleavage site selection.

Based on the kinetic characterization performed here, the substrate selectivity of PRORP2 and 3 are more similar to one another than to PRORP1, consistent with their higher sequence similarity and knockout data that suggest a redundant function for PRORP2 and 3 within the nucleus (Gutmann et al. 2012). Furthermore, the fidelity of PRORP2 is more similar to PRORP3 and, interestingly, both enzymes share higher activity toward the SL substrate than PRORP1 ($k_{obs} > 12$ -fold) (Supplemental Table S2).

This suggests that the nuclear PRORPs have subtle but distinct differences in substrate recognition as compared to the organellar PRORP1. The overlapping substrate specificities of *Arabidopsis* PRORP enzymes suggest that they are in the early stages of diversification, which may be the result of relatively recent gene duplication events (Lynch and Conery 2000; Gobert et al. 2010). It is interesting to note that *Arabidopsis* encodes four variants of the nuclease that catalyzes 3' end pre-tRNA maturation, tRNase Z (Canino et al. 2009). These enzymes are differentially localized, but only the chloroplast-localized tRNase Z knockout is lethal, suggesting several isozymes have a redundant function, as with PRORP2 and 3. The abundance of tRNase Z enzymes in *Arabidopsis* is attributed to differential cellular compartmental localization, differential expression, and/or tissue specific expression (Canino et al. 2009). The data available suggest that this is also the case with PRORP enzymes.

MATERIALS AND METHODS

Enzyme and substrate preparation

PRORP constructs ($\Delta 76$ PRORP1, full-length PRORP2, and $\Delta 9$ PRORP3 [Gobert et al. 2010]) were cloned, expressed and purified from *E. coli* as previously described (Howard et al. 2012). The concentrations of PRORP1, 2 and 3 were determined by absorbance using extinction coefficients in the native state at 280 nm of 84,630 M⁻¹ cm⁻¹, 91,300 M⁻¹ cm⁻¹, and 84,700 M⁻¹ cm⁻¹, respectively. Purified enzymes were aliquoted, flash-frozen, and stored at -80°C in 20 mM MOPS pH 7.8, 100 mM NaCl, and 1 mM TCEP. Truncation of PRORP1 was required to obtain soluble protein. However, full-length PRORP2 expressed in *E. coli* is observed mainly in the soluble fraction. A $\Delta 20$ amino acid truncation of PRORP2 at the N-terminus (the comparable truncation to $\Delta 76$ PRORP1) has comparable activity to full-length PRORP2 (Karasik et al. 2016).

Pre-tRNA substrates were prepared by in vitro transcription catalyzed by T7 polymerase (Milligan and Uhlenbeck 1989; Brunelle and Green 2013). Substrate sequences were retrieved from the *A. thaliana* genomic tRNA database (Michaud et al. 2011). The DNA template for transcription was created by PCR amplification of DNA purchased from Life Technologies GeneArt. To generate fluorescein-labeled pre-tRNA substrates, transcription reactions were performed in the presence of guanosine monophosphorothioate and the 5' phosphorothioate was reacted with 5-(iodoacetamido)fluorescein to label the 5' end, as previously described (Rueda et al. 2005). The pre-tRNA substrates were purified by denaturing PAGE (12%). The discriminator base on the SL substrate was removed to mitigate miscleavage products catalyzed by PRORP2 and 3. The proposed tRNA secondary structures were generated with tRNAscan-SE 1.21 (Schattner et al. 2005).

Multiple-turnover assays

Multiple-turnover (MTO) reactions were performed in a 96-well plate format using a fluorescence anisotropy (FA) assay (as described in Liu et al. 2014). Standard reaction conditions (25°C, 30 mM MOPS pH 7.8, 150 mM NaCl, 1 mM TCEP, and 1 mM MgCl₂) were used with an enzyme concentration of 20 nM (PRORP1 and PRORP3) or 80 nM (PRORP2). Higher concentrations of PRORP2 were required to obtain consistent $v_0/[E]$ values at a given substrate concentration. The concentration of fluorescently labeled pre-tRNA was held constant at 40 nM in the reactions while the concentration of unlabeled pre-tRNA substrate was varied. The ratio of labeled to unlabeled substrate did not alter the measured initial rates (data not shown). Black Corning half-area 96-well plates were used with a final reaction volume of 40 μ L per well. Initial rates were calculated from the linear decrease in anisotropy (Liu et al. 2014). The steady-state kinetic parameters were calculated from a fit of the Michaelis-Menten equation (Equation 1) to the concentration-dependence of the initial rates using KaleidaGraph (Synergy Software). These kinetic parameters encompass both incorrect and correct cleavage product formation. The reported kinetic parameters and standard error were determined by fitting Equation 1 to the concentration-dependence of the initial rates.

$$\frac{v_0}{[E]} = \frac{k_{\text{cat}}[S]}{K_M + [S]} \quad (1)$$

Anisotropy binding assays

Binding assays were performed as described previously (Howard et al. 2012). Briefly, the concentration of fluorescein-labeled pre-tRNA was maintained at 20 nM, while the concentration of PRORP was varied (0.005–20 μ M). Binding experiments were performed in 20 mM MOPS pH 7.8, 300 mM NaCl, 1 mM TCEP, and 1 mM CaCl₂ in a 96-well plate format. PRORP1 can bind but not cleave substrates in CaCl₂ (Howard et al. 2012). Fluorescence anisotropy was measured at an excitation wavelength of 488 nm and an emission wavelength of 535 nm. The observed anisotropies in binding assays where the total fluorescence intensity increased >15% upon PRORP addition (*B. subtilis* substrates containing 1-, 2-, 3-, and 4-nt leaders) were corrected (Lakowicz 2006). Equation 2 was fit to the dependence of the anisotropy on the protein concentration where A is the observed anisotropy, A_0 is the initial anisotropy, ΔA is the total change in anisotropy, $[P]$ is the concentration of PRORP, and K_D is the apparent dissociation constant.

$$A = A_0 + \frac{\Delta A[P]}{[P] + K_D} \quad (2)$$

Single-turnover assays

For single-turnover (STO) reactions, the enzyme and pre-tRNA concentrations were 5 μ M and 30 nM, respectively,

unless otherwise noted. Reactions were performed in standard conditions (see MTO), initiated by addition of enzyme, and quenched at specified time points (0–1200 sec) with an equal volume of 100 mM EDTA, 6 M urea, 0.1% bromophenol blue, 0.1% xylene cyanol, and 2 $\mu\text{g}/\mu\text{L}$ yeast tRNA. The fluorescently labeled 5' leader product was separated from pre-tRNA by electrophoresis on 20% or 22.5% denaturing PAGE gel. Gels were visualized using a Typhoon 9410 scanner and the fraction product quantified using ImageQuant 5.2 software. The observed single-turnover rate constant was calculated from a fit of a first order exponential equation to the data using KaleidaGraph fitting software (Equation 3), where A is the end point, B is the amplitude, k is the observed rate constant, and t is time. The STO assays with *Bacillus subtilis* RNase P were performed at 25°C with 1.8 μM bacterial RNase P, 50 mM Tris pH 7.4, 100 mM KCl, 75 mM NaCl, 10 mM DTT, and 3 mM MgCl_2 . The *B. subtilis* RNase P was prepared as previously described (Hsieh and Fierke 2009). The alkaline ladder was produced by the incubation of a given pre-tRNA in 10 mM NaOH and 1 mM EDTA at 95°C for 2 min. Free fluorescein runs as a blur near the second nt (Fig. 6). The miscleavage product (4 nt) does not align with the 4-nt ladder product (Fig. 6). This may be a result of running near the gel front in combination with differences in the alkaline cleavage products (3' Phosphate) and RNase P products (3' hydroxyl). STO miscleavage kinetics catalyzed by the PRORPs were analyzed as described in Loria and Pan (1998) and Zahler et al. (2005). Briefly, the observed rate constants for both correct and miscleaved products are obtained from a single-exponential fit to the data (Equation 3). The resulting amplitude (A_c or A_m) for each respective fit is multiplied by the observed rate constant ($k_{\text{obs},c}$ or $k_{\text{obs},m}$) to obtain k_c and k_m (Equations 4 and 5).

$$\text{Fraction product} = A - B(e^{-kt}) \quad (3)$$

$$k_c = k_{\text{obs},c} (A_c) \quad (4)$$

$$k_m = k_{\text{obs},m} (A_m) \quad (5)$$

Primer extension

Processing of the Phe-Nuc miscleavage product catalyzed by PRORP1 and PRORP3 was measured by primer extension using Omniscript (QIAGEN) reverse transcriptase (RTase). Substrate was incubated with the PRORPs under standard conditions. Time points were mixed 1:1 with a quench solution containing 10 mM Tris pH 8.0, 50 mM EDTA, and 8 M urea. RNA was repurified by phenol/chloroform extraction and ethanol precipitation. The ladder was generated by alkaline hydrolysis of substrate in 10 mM NaOH and 1 mM EDTA at 95°C for 10 min, then quenched with HCl. The time points, ladder, and substrate were each used to template reverse transcription (RT) reactions. Each template was melted in the presence of an oligo-dNTP primer (IDT, 5'-FAM-

CCCAACTGAGCTATCC-3') at 95°C for 3 min, then cooled to 37°C. The primed-RNA was then mixed 1:1 with a master mix containing Omniscript RT buffer, dNTPs, SUPERase•In (Thermo Fisher), and Omniscript RTase, and incubated at 37°C for 60 min. Glycerol was added for gel loading and products were separated using denaturing PAGE (22.5%) and then fluorescence of the fluorescein label was imaged with a Typhoon 9410 scanner. The substrate and product bands were quantified using ImageQuant 5.2 software. The fraction miscleaved was calculated with $[M_{-1}/(C_0 + M_{-1} + \text{Substrate})]$. As has been previously reported, some nontemplated nucleotide addition by the RTase was observed (Chen and Patton 2001).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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REFERENCES

- Beebe JA, Fierke CA. 1994. A kinetic mechanism for cleavage of precursor tRNA^{Asp} catalyzed by the RNA component of *Bacillus subtilis* ribonuclease P. *Biochemistry* **33**: 10294–10598.
- Bhaskaran H, Taniguchi T, Suzuki T, Perona JJ. 2014. Structural dynamics of a mitochondrial tRNA possessing weak thermodynamic stability. *Biochemistry* **53**: 1456–1465.
- Brännvall M, Kikovska E, Wu S, Kirsebom LA. 2007. Evidence for induced fit in bacterial RNase P RNA-mediated cleavage. *J Mol Biol* **372**: 1149–1164.
- Brunelle JL, Green R. 2013. In vitro transcription from plasmid or PCR-amplified DNA. *Methods Enzymol* **530**: 101–114.
- Canino G, Bocian E, Barbezier N, Echeverría M, Forner J, Binder S, Marchfelder A. 2009. *Arabidopsis* encodes four tRNase Z enzymes. *Plant Physiol* **150**: 1494–1502.
- Chen D, Patton JT. 2001. Reverse transcriptase adds nontemplated nucleotides to cDNAs during 5'-RACE and primer extension. *Biotechniques* **30**: 574–580, 582.
- Crary SM, Niranjankumari S, Fierke CA. 1998. The protein component of *Bacillus subtilis* ribonuclease P increases catalytic efficiency by enhancing interactions with the 5' leader sequence of pre-tRNA^{Asp}. *Biochemistry* **37**: 9409–9416.
- Emanuelsson O, Brunak S, von Heijne G, Nielsen H. 2007. Locating proteins in the cell using TargetP, SignalP and related tools. *Nat Protoc* **2**: 953–971.
- Gobert A, Gutmann B, Taschner A, Gössringer M, Holzmann J, Hartmann RK, Rossmann W, Giegé P. 2010. A single *Arabidopsis* organellar protein has RNase P activity. *Nat Struct Mol Biol* **17**: 740–744.

- Gobert A, Pinker F, Fuchsbaauer O, Gutmann B, Boutin R, Roblin P, Sauter C, Giegé P. 2013. Structural insights into protein-only RNase P complexed with tRNA. *Nat Commun* **4**: 1353.
- Goldfarb KC, Borah S, Cech TR. 2012. RNase P branches out from RNP to protein: organelle-triggered diversification? *Genes Dev* **26**: 1005–1009.
- Gutmann B, Gobert A, Giegé P. 2012. PRORP proteins support RNase P activity in both organelles and the nucleus in *Arabidopsis*. *Genes Dev* **26**: 1022–1027.
- Hartmann E, Hartmann RK. 2003. The enigma of ribonuclease P evolution. *Trends Genet* **19**: 561–569.
- Holzmann J, Frank P, Löffler E, Bennett KL, Gerner C, Rossmannith W. 2008. RNase P without RNA: identification and functional reconstitution of the human mitochondrial tRNA processing enzyme. *Cell* **135**: 462–474.
- Howard MJ, Lim WH, Fierke CA, Koutmos M. 2012. Mitochondrial ribonuclease P structure provides insight into the evolution of catalytic strategies for precursor-tRNA 5' processing. *Proc Natl Acad Sci* **109**: 16149–16154.
- Howard MJ, Liu X, Lim WH, Klemm BP, Fierke CA, Koutmos M, Engelke DR. 2013. RNase P enzymes: divergent scaffolds for a conserved biological reaction. *RNA Biol* **10**: 909–914.
- Howard MJ, Klemm BP, Fierke CA. 2015. Mechanistic studies reveal similar catalytic strategies for phosphodiester bond hydrolysis by protein-only and RNA-dependent Ribonuclease P. *J Biol Chem* **290**: 13454–13464.
- Hsieh J, Fierke CA. 2009. Conformational change in the *Bacillus subtilis* RNase P holoenzyme–pre-tRNA complex enhances substrate affinity and limits cleavage rate. *RNA* **15**: 1565–1577.
- Imai T, Nakamura T, Maeda T, Nakayama K, Gao X, Nakashima T, Kakuta Y, Kimura M. 2014. Pentatricopeptide repeat motifs in the processing enzyme PRORP1 in *Arabidopsis thaliana* play a crucial role in recognition of nucleotide bases at TψC loop in precursor tRNAs. *Biochem Biophys Res Commun* **450**: 1541–1546.
- Karasik A, Shanmuganathan A, Howard MJ, Fierke CA, Koutmos M. 2016. Nuclear protein-only Ribonuclease P2 structure and biochemical characterization provide insight into the conserved properties of tRNA 5' end processing enzymes. *J Mol Biol* **428**: 26–40.
- Lai LB, Bernal-Bayard P, Mohannath G, Lai SM, Gopalan V, Vioque A. 2011. A functional RNase P protein subunit of bacterial origin in some eukaryotes. *Mol Genet Genomics* **286**: 359–369.
- Lakowicz RJ. 2006. *Principles of fluorescence spectroscopy*. Springer, New York.
- Lange A, Mills RE, Lange CJ, Stewart M, Devine SE, Corbett AH. 2007. Classical nuclear localization signals: definition, function, and interaction with importin α . *J Biol Chem* **282**: 5101–5105.
- Lechner M, Rossmannith W, Hartmann RK, Thölken C, Gutmann B, Giegé P, Gobert A. 2015. Distribution of ribonucleoprotein and protein-only RNase P in eukarya. *Mol Biol Evol* **32**: 3186–3193.
- Liu X, Chen Y, Fierke CA. 2014. A real-time fluorescence polarization activity assay to screen for inhibitors of bacterial ribonuclease P. *Nucleic Acids Res* **42**: e159.
- Loria A, Pan T. 1998. Recognition of the 5' leader and the acceptor stem of a pre-tRNA substrate by the ribozyme from *Bacillus subtilis* RNase P. *Biochemistry* **37**: 10126–10133.
- Lynch M, Conery JS. 2000. The evolutionary fate and consequences of duplicate genes. *Science* **290**: 1151–1155.
- Michaud M, Cognat V, Duchêne AM, Maréchal-Drouard L. 2011. A global picture of tRNA genes in plant genomes. *Plant J* **66**: 80–93.
- Milligan JF, Uhlenbeck OC. 1989. Synthesis of small RNAs using T7 RNA polymerase. *Methods Enzymol* **180**: 51–62.
- Niranjankumari S, Stams T, Crary SM, Christianson DW, Fierke CA. 1998. Protein component of the ribozyme ribonuclease P alters substrate recognition by directly contacting precursor tRNA. *Proc Natl Acad Sci* **95**: 15212–15217.
- Pavlova LV, Gossringer M, Weber C, Buzet A, Rossmannith W, Hartmann RK. 2012. tRNA processing by protein-only versus RNA-based RNase P: kinetic analysis reveals mechanistic differences. *ChemBiochem* **13**: 2270–2276.
- Placido A, Sieber F, Gobert A, Gallerani R, Giegé P, Maréchal-Drouard L. 2010. Plant mitochondria use two pathways for the biogenesis of tRNA^{His}. *Nucleic Acids Res* **38**: 7711–7717.
- Reiter NJ, Osterman A, Torres-Larios A, Swinger KK, Pan T, Mondragón A. 2010. Structure of a bacterial ribonuclease P holoenzyme in complex with tRNA. *Nature* **468**: 784–789.
- Rueda D, Hsieh J, Day-Storms JJ, Fierke CA, Walter NG. 2005. The 5' leader of precursor tRNA^{Asp} bound to the *Bacillus subtilis* RNase P holoenzyme has an extended conformation. *Biochemistry* **44**: 16130–16139.
- Schattner P, Brooks AN, Lowe TM. 2005. The tRNAscan-SE, snoscan and snoGPS web servers for the detection of tRNAs and snoRNAs. *Nucleic Acids Res* **33**: W686–W689.
- Steitz TA, Steitz JA. 1993. A general two-metal-ion mechanism for catalytic RNA. *Proc Natl Acad Sci* **90**: 6498–6502.
- Sugita C, Komura Y, Tanaka K, Kometani K, Satoh H, Sugita M. 2014. Molecular characterization of three PRORP proteins in the moss *Physcomitrella patens*: nuclear PRORP protein is not essential for moss viability. *PLoS One* **9**: e108962.
- Sun L, Campbell FE, Yandek LE, Harris ME. 2010. Binding of C5 protein to P RNA enhances the rate constant for catalysis for P RNA processing of pre-tRNAs lacking a consensus (+ 1)/C(+ 72) pair. *J Mol Biol* **395**: 1019–1037.
- Taschner A, Weber C, Buzet A, Hartmann RK, Hartig A, Rossmannith W. 2012. Nuclear RNase P of *Trypanosoma brucei*: a single protein in place of the multicomponent RNA-protein complex. *Cell Rep* **2**: 19–25.
- Vilardo E, Rossmannith W. 2015. Molecular insights into HSD10 disease: impact of SDR5C1 mutations on the human mitochondrial RNase P complex. *Nucleic Acids Res* **43**: 5112–5119.
- Vilardo E, Nachbagauer C, Buzet A, Taschner A, Holzmann J, Rossmannith W. 2012. A subcomplex of human mitochondrial RNase P is a bifunctional methyltransferase—extensive moonlighting in mitochondrial tRNA biogenesis. *Nucleic Acids Res* **40**: 11583–11593.
- Walker SC, Engelke DR. 2006. Ribonuclease P: the evolution of an ancient RNA enzyme. *Crit Rev Biochem Mol Biol* **41**: 77–102.
- Zahler NH, Christian EL, Harris ME. 2003. Recognition of the 5' leader of pre-tRNA substrates by the active site of ribonuclease P. *RNA* **9**: 734–745.
- Zahler NH, Sun L, Christian EL, Harris ME. 2005. The pre-tRNA nucleotide base and 2'-hydroxyl at N(–1) contribute to fidelity in tRNA processing by RNase P. *J Mol Biol* **345**: 969–985.
- Zhou W, Karcher D, Fischer A, Maximova E, Walther D, Bock R. 2015. Multiple RNA processing defects and impaired chloroplast function in plants deficient in the organellar protein-only RNase P enzyme. *PLoS One* **10**: e0120533.

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