SUPPLEMENTARY DATA

Substrate recognition and cleavage-site selection by a single-subunit protein-only RNase P

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Supplementary Table S1. Oligonucleotides used for cloning and site-directed mutagenesis of PRORP3.

Amino acid substitution	Forward	Reverse
none[a]	GGCTCTAGACCATGGCTGGTACTGAT	CGGCTCGAGTGAACTCTGCCTTGTA
T113S	CTAATGAATCATCTGTCTCTGCAGTTG CACGACTAG	CTAGTCGTGCAACTGCAGAGACAGAT GATTCATTAG
T113N	CTAATGAATCATCTGTCAATGCAGTTG CACGACTAG	CTAGTCGTGCAACTGCATTGACAGAT GATTCATTAG
R145N	GTGTATCGGTCCCTAATCTGAGAACTT ATGC	GCATAAGTTCTCAGATTAGGGACCGA TACAC
R145D	GTGTATCGGTCCCTGATCTGAGAACTT ATGC	GCATAAGTTCTCAGATCAGGGACCGA TACAC

[a]Primer pair used for the cloning of the *A. thaliana* wild-type PRORP3 cDNA.

Supplementary Table S2. Oligonucleotides used for site-directed mutagenesis and (PCR) cloning of RNA-substrate templates.

RNA substrate	Forward	Reverse
U_1-A_{72}[a]	TAGGATTTTCCCTTTCTCGGGAGTAGCTCAGTC CAAGTCCCGTCTCCCGATCCAGTCACCGGATGTGC	GACTGAGCTACTCCCGAGAAAGGGAAAATCCTA GCACATCCGGTGACTGGATCGGGAGACGGGACTTG
U_{-1}	CTATAGGATTTTCCCTTTTGCGGGAGTAGCTCAGTCG	CGACTGAGCTACTCCCGCAAAAGGGAAAATCCTATAG
G_{-1}, A_{73}[a]	TATAGGATTTTCCCTTTGGCGGGAGTAGCTCAGTCG CAAGTCCCGTCTCCCGCACCAGTCACCGGATGTGC	CGACTGAGCTACTCCCGCCAAAGGGAAAATCCTATA GCACATCCGGTGACTGGTGCGGGAGACGGGACTTG
A_{-1}, A_{73}[a]	CTATAGGATTTTCCCTTTAGCGGGAGTAGCTCAGTCG CAAGTCCCGTCTCCCGCACCAGTCACCGGATGTGC	CGACTGAGCTACTCCCGCTAAAGGGAAAATCCTATAG GCACATCCGGTGACTGGTGCGGGAGACGGGACTTG
A_{73}	CAAGTCCCGTCTCCCGCACCAGTCACCGGATGTGC	GCACATCCGGTGACTGGTGCGGGAGACGGGACTTG
G_{18}→A_{18}	GCGGGAGTAGCTCAGTCAGTAGAGCACGACCTTGC	GCAAGGTCGTGCTCTACTGACTGAGCTACTCCCGC
G_{19}→A_{19}, C_{56}→U_{56}[a]	TCGGGGTCGCGGGTTTAAGTCCCGTCTCCCG GGAGTAGCTCAGTCGATAGAGCACGACCTTGC	CGGGAGACGGGACTTAAACCCGCGACCCCGA GCAAGGTCGTGCTCTATCGACTGAGCTACTCC
C_{56}→U_{56}	TCGGGGTCGCGGGTTTAAGTCCCGTCTCCCG	CGGGAGACGGGACTTAAACCCGCGACCCCGA
A_{57}→C_{57}	GGTCGCGGGTTCCAGTCCCGTCTCC	GGAGACGGGACTGGAACCCGCGACC
U_{65}→C_{65}[b]	GGTTCAAGTCCCGCCTCCCGCTCCAGT	ACTGGAGCGGGAGGCGGGACTTGAACC
$Aa_{b1}T$[c]	TTCCCTTTCGCGGGAGAGCGGGTTCAAGTC	GACTTGAACCCGCTCTCCCGCGAAAGGGAA
$Aa_{b4}T$[d]	TTCCCTTTCGCGGGAGAGCTGCGGGTTCAAGTC GGAATTGTGAGCGGATAACA	GGTCTGCTTCAGTAAGCCAG GACTTGAACCCGCAGCTCTCCCGCGAAAGGGAA
$Aa_{b9}T$[d]	TTCCCTTTCGCGGGAGCCAGCTCCTGCGGGTTCAAGTC GGAATTGTGAGCGGATAACA	GGTCTGCTTCAGTAAGCCAG GACTTGAACCCGCAGGAGCTGGCTCCCGCGAAAGGGAA
AaT	AATTCGAGCTCGCCTAATACGACTCACTATAGGATTTT CCCTTTCGCGGGAGGCGGGTTCAAGTCCCGCCTCCCG CTCCAGTCACCGGATGTGCTTTCCGGTCTGATGAGTCC GTGAGGACGAAACTGGTATG	GATCCATACCAGTTTCGTCCTCACGGACTCATCAGACC GGAAAGCACATCCGGTGACTGGAGCGGGAGGCGGG ACTTGAACCCGCCTCCCGCGAAAGGGAAAATCCTATA GTGAGTCGTATTAGGCGAGCTCG
Aa_{-2bp}[a]	GTTCAAGTCCCGTCTCGCTCCAGTCACCG ACTATAGGATTTTCCCTTTCGCGAGTAGCTCAGTCGG	CGGTGACTGGAGCGAGACGGGACTTGAAC CCGACTGAGCTACTCGCGAAAGGGAAAATCCTATAGT
Aa_{+2bp}[a]	TCAAGTCCCGTCTCCGGCGCTCCAGTCACC TAGGATTTTCCCTTTCGCGCCGGAGTAGCTCAGTC	GGTGACTGGAGCGCCGGAGACGGGACTTGA GACTGAGCTACTCCGGCGCGAAAGGGAAAATCCTA

[a]Primer pairs for 2 rounds of site-directed mutagenesis.

[b]Used to generate the plasmid encoding pre-tRNA^{Gly}-U_{65}→C_{65}, used as a template for further mutagenesis only.

[c]Plasmid encoding pre-tRNA^{Gly}-U_{65}→C_{65} was used as a DNA template in the mutagenesis.

[d]Primer pairs to produce 2 overlapping PCR products from plasmid encoding pre-tRNA^{Gly}-U_{65}→C_{65}.

Supplementary Table S3. Oligonucleotides used for "inside-out" PCR mutagenesis of RNA-substrate templates.

RNA substrate	Forward primer	Reverse primer
7-nt leader	AGGACGAAACGGTACCCGGTACCGTCCCCTTTC GCGGGAGTAGCTCAGTCGGTAGAGCACGACC	CACGGACTCATCAGCCCTTTCGCTCCTATAGTGAGTC GTATTAGGGCGAGCTCGAATTCGTAATCATGG
4-nt leader	AGGACGAAACGGTACCCGGTACCGTCTTTCGCG GGAGTAGCTCAGTCGGTAGAGCACGACC	CACGGACTCATCAGTTTCGCGGCTCCTATAGTGAGT CGTATTAGGGCGAGCTCGAATTCGTAATCATGG
2-nt leader	AGGACGAAACGGTACCCGGTACCGTCCCGCGG GAGTAGCTCAGTCGGTAGAGCACGACC	CACGGACTCATCAGCCGCGGGACTCCTATAGTGAGT CGTATTAGGGCGAGCTCGAATTCGTAATCATGG
1-nt leader	AGGACGAAACGGTACCCGGTACCGTCCGCGGG AGTAGCTCAGTCGGTAGAGCACGACC	CACGGACTCATCAGCGCGGGAGCTCCTATAGTGAGT CGTATTAGGGCGAGCTCGAATTCGTAATCATGG

Supplementary Table S4. Oligonucleotides used to prepare RNA-substrate templates by PCR.

RNA substrate	Forward primer	Reverse primer
(mature) CCA[a]	CTCGAGTAATACGACTCACTATAGG	TGGAGCGGGAGACGGGACTT
no trailer[a]	CTCGAGTAATACGACTCACTATAGG	AGCGGGAGACGGGACTT
40-nt trailer[a]	CTCGAGTAATACGACTCACTATAGG	TCCTCACGGACTCATCAG
ΔAc[b]	TAATACGACTCACTATAGG	TATTGGAGCGGGAGACG
ΔD[b]	TAATACGACTCACTATAGG	TATTGGAGCGGGAGACG
$Aa_{b9}T$-U_{54}→C_{54}[c]	CTCGAGTAATACGACTCACTATAGGATT TTCCCTTTCGCGGGAGCCAGCTCCTG	GACTGGAGCGGGAGGCGGGACTTGAG CCCGCAGGAGCTGGC
$Aa_{b9}T$-U_{55}→C_{55}[c]	CTCGAGTAATACGACTCACTATAGGATT TTCCCTTTCGCGGGAGCCAGCTCCTG	GACTGGAGCGGGAGGCGGGACTTGGA CCCGCAGGAGCTGGC
$Aa_{b9}T$-C_{56}→U_{56}[c]	CTCGAGTAATACGACTCACTATAGGATT TTCCCTTTCGCGGGAGCCAGCTCCTG	GACTGGAGCGGGAGGCGGGACTTAAA CCCGCAGGAGCTGGC
$Aa_{b9}T$-A_{57}→G_{57}[c]	CTCGAGTAATACGACTCACTATAGGATT TTCCCTTTCGCGGGAGCCAGCTCCTG	GACTGGAGCGGGAGGCGGGACTCGAA CCCGCAGGAGCTGGC
$Aa_{b9}T$-A_{57}→C_{57}[c]	CTCGAGTAATACGACTCACTATAGGATT TTCCCTTTCGCGGGAGCCAGCTCCTG	GACTGGAGCGGGAGGCGGGACTGGAA CCCGCAGGAGCTGGC
$Aa_{b9}T$-A_{57}→U_{57}[c]	CTCGAGTAATACGACTCACTATAGGATT TTCCCTTTCGCGGGAGCCAGCTCCTG	GACTGGAGCGGGAGGCGGGACTAGAA CCCGCAGGAGCTGGC
$Aa_{b9}T$-A_{58}→G_{58}[c]	CTCGAGTAATACGACTCACTATAGGATT TTCCCTTTCGCGGGAGCCAGCTCCTG	GACTGGAGCGGGAGGCGGGACCTGAA CCCGCAGGAGCTGGC
$Aa_{b9}T_{CCUUUUA}$[c]	CTCGAGTAATACGACTCACTATAGGATT TTCCCTTTCGCGGGAGCCAGCTCCTG	GACTGGAGCGGGAGGCGGGTAAAAGG CCCGCAGGAGCTGGC
$Aa_{+2bp\ b9}T$[c]	CTCGAGTAATACGACTCACTATAGGATT TTCCCTTTCGCGCCGGAGCCAGCTCCTG	GACTGGAGCGCCGGAGGCGGGACTTG AACCCGCAGGAGCTGGC
$Aa_{b9}T_{+2bp}$[c]	CTCGAGTAATACGACTCACTATAGGATT TTCCCTTTCGCGGGAGCCAGCTCCTG	GACTGGAGCGGGAGGCGCCGGACTTG AACCGGCGCAGGAGCTGGC
$Aa_{-2bp\ b9}T_{+2bp}$[c]	CTCGAGTAATACGACTCACTATAGGATT TTCCCTTTCGCGAGCCAGCTCCTG	GACTGGAGCGAGGCGCCGGACTTGAAC CGGCGCAGGAGCTGGC
$Aa_{b9}T_{4loop}$[c]	CTCGAGTAATACGACTCACTATAGGATT TTCCCTTTCGCGGGAGCCAGCTCCTG	GACTGGAGCGGGAGGCGGGTTTCCCCG CAGGAGCTGGC
Aa_{+4bp}[b]	CTCGAGTAATACGACTCACTATAGG	GACTGGAGCGCGCCGGAGA
Aa_{+m3GC}[b]	CTCGAGTAATACGACTCACTATAGG	GACTGGAGCGAGCCGGAGA
Aa_{+m3AU}[b]	CTCGAGTAATACGACTCACTATAGG	GACTGGAATAAGCCGGAGA
G_{-1}-C_{73}[d]	CTCGAGTAATACGACTCACTATAGG	GACTGGGGCGGGAGA
U_{-1}-A_{73}[e]	CTCGAGTAATACGACTCACTATAGG	GACTGGTGCGGGAGA
Aa_{+3AU}[a]	CGAGTAATACGACTCACTATAGGATTTT CCCTTTCAATGCGGGAGTAGC	GACTGGAAATGCGGGAGAC

[a]Plasmid pSBpt3'hh was used as a template.

[b]Oligonucleotide template listed in Supplementary Table S6.

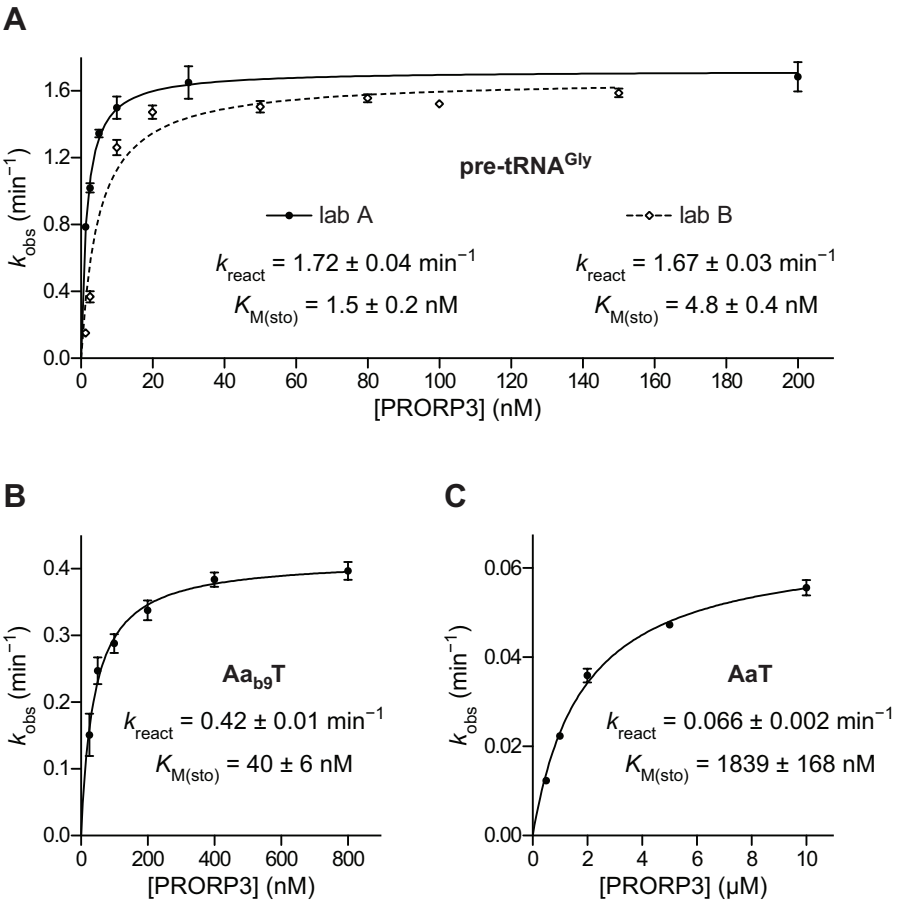
[c]Overlapping primers (no extra template).

[d]Pre-$tRNA^{Gly}$-G_{-1} was used as a template.

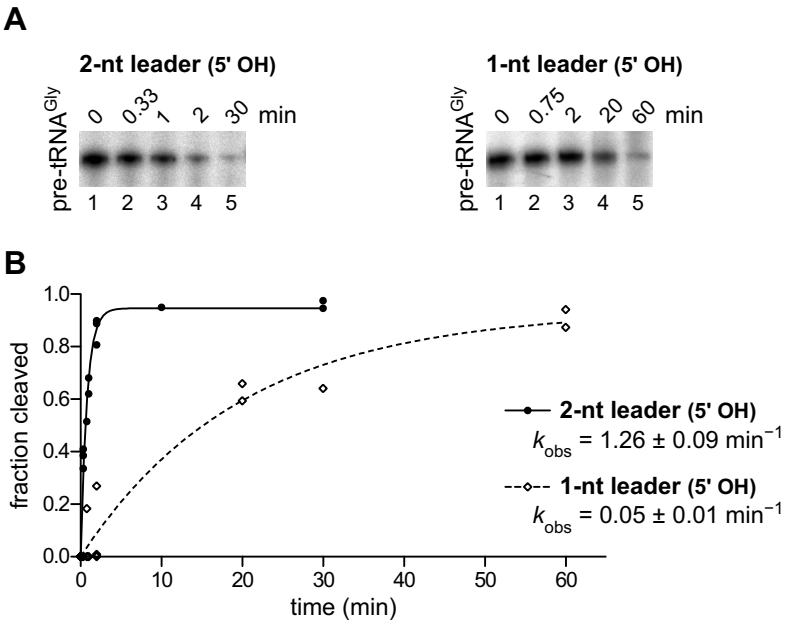
[e]Pre-$tRNA^{Gly}$-U_{-1} was used as a template.

Supplementary Table S5. Oligonucleotide templates used for PCR of RNA-substrate templates.

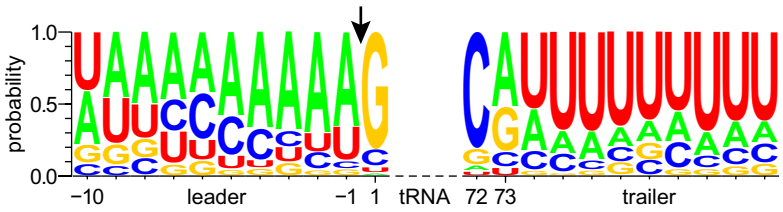
RNA substrate	Oligonucleotide template
Aa_{+4bp} & Aa_{+m3GC}	TAATACGACTCACTATAGGATTTTCCCTTTCGCGCGCCGGAGTAGCTCAGTCGGTAGAGC ACGACCTTGCCAAGGTCGGGGTCGCGGGTTCAAGTCCCGTCTCCGGCGCGCTCCAGTC
Aa_{+m3AU}	TAATACGACTCACTATAGGATTTTCCCTTTCATACGCCGGAGTAGCTCAGTCGGTAGAGCA CGACCTTGCCAAGGTCGGGGTCGCGGGTTCAAGTCCCGTCTCCGGCGTATTCCAGTC
ΔD	TAATACGACTCACTATAGGATTTTCCCTTTCGCGGGAGTAGACCGACCTTGCCAAGGTCG GGTCGCGGGTTCAAGTCCCGTCTCCCGCTCCAATA
ΔAC	TAATACGACTCACTATAGGATTTTCCCTTTCGCGGGAGTAGCTCAGTCGGTAGAGCACCTT CGGGGGGTCGCGGGTTCAAGTCCCGTCTCCCGCTCCAATA



Supplementary Figure S1. Single-turnover cleavage kinetics of representative substrates. First-order rate constants of cleavage (k_{obs}) obtained at different PRORP3 concentrations were plotted against the enzyme concentration and fit by nonlinear regression to a "Michaelis-Menten-like" enzyme kinetics model to derive the maximal rate constant k_{react} and the single-turnover Michaelis constant $K_{M(sto)}$. Data points represent the mean of at least 3 replicates with SEM (error bars display only if SEM exceeds the size of the data point in the graph). (**A**) Kinetic analysis of pre-tRNA^{Gly} cleavage by PRORP3 independently performed by the two labs. (**B**) Kinetic analysis of the cleavage of the minimal substrate $Aa_{b9}T$ by PRORP3. (**C**) Kinetic analysis of the cleavage of the stem-loop substrate AaT by PRORP3.



Supplementary Figure S2. Cleavage analysis of pre-tRNA^{Gly} variants with a short 5'-hydroxyl-end leader. (**A**) Unlabeled pre-tRNA^{Gly} variants with a 5'-hydroxyl-end leader of 1 or 2 nt were cleaved with PRORP3 under presumably enzyme-saturated conditions (80 nM). Aliquots of the reactions were withdrawn at indicated times, and the RNA ^{32}P-5'-end labeled and resolved by denaturing PAGE. The panels show the substrate RNA remaining after the reaction time indicated. (**B**) First-order rate constants of cleavage (k_{obs}; best-fit values ± standard error) for the two substrates were derived from the data of three experiments each. Individual data points are shown ("substrate remaining" converted to "fraction cleaved"; note: in the three replicate experiments some sampling time points differed).



Supplementary Figure S3. Nucleotide frequencies of the 5'-leader and 3'-trailer sequences of the nucleus-encoded pre-tRNAs of *A. thaliana*. Nucleotides upstream of the 5' end and downstream of the 3' end of the mature tRNA, including the first base pair of the tRNAs' nucleotides 1 and 72, and the discriminator position 73, were aligned, and a logo derived from the alignment of the 598 sequences. An arrow indicates the canonical RNase P cleavage site.

Supplementary Table S6. Base-pair frequencies at the RNase P cleavage site of the nucleus-encoded pre-tRNAs of *A. thaliana*.

Base-paired positions[a]	G-C/C-G bp	A-U/U-A/G-U/U-G bp	G-C/C-G as 1st or 2nd upstream bp[b]
+1 - 72	92.0%	7.0%	
−1 - 73	1.0%	22.9%	
−2 - 74 −1 - 73	0.0%	8.5%	1.5%
−3 - 75 −2 - 74 −1 - 73	0.0%	3.5%	0.4%
−4 - 76 −3 - 75 −2 - 74 −1 - 73	0.0%	1.8%	0.0%

[a]Numbering according to convention with the exception of positions 74–76, which do not indicate the CCA end of the mature tRNA, but the first 3 nt of the trailer of the pre-tRNA (see also Supplementary Figure S3); nucleotides of the 5' leader carry negative numbers starting from the nucleotide closest to the RNase P cleavage site with −1.

[b]Extensions of the acceptor stem with a single G-C base pair at the first or second upstream position; the other base pairs of the extension are either A-U or G-U.